Food Research

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INSIDE SCIENCE

The Vital Story of VITAMIN A

by Science Writer

We live in a golden age of the sciences!

In the science of nutrition discoveries of food elements and their action have resulted in the promise of better health for us all.

Let's consider vitamin A as an example.

A Capsule History. 1913 was the year. Two teams working independently with animal feeding experiments discovered that a diet which was good in all other respects but which varied only in the source of fat caused young animals to thrive or sicken as the fat was varied. The work of these teams (McCollum and Davis; Osborne and Mendel) led to the discovery of the food factor which we know as vitamin A.

Composition and Action. A pale yellow, oil-soluble substance, vitamin A is expressed in the chemist's shorthand as $C_{20}H_{29}OH$. It is necessary in the diets of men and animals to promote the body's growth and development. Vitamin A is a vital requirement in guarding the health of the eyes and skin, and for resistance against infection. It helps maintain the health of mucous membranes and other specialized epithelial and glandular tissues.

Vitamin A enters the body as a constituent of food. Or it may be created within the body when a precursor of vitamin A is eaten. A precursor is a food element with potential vitamin A *value* but no actual vitamin A *content*. For instance, many green leaves have a high vitamin A value but, paradoxically, contain no actual vitamin A. The body converts the precursor into the vitamin. The most common precursor of vitamin A is beta carotene, the natural yellow coloring of many foods, such as dairy products, cereal grains, carrots, alfalfa, etc.

When consumed in food vitamin A is absorbed primarily in the healthy intestine, is esterified in the intestine's walls and is carried as an ester in the lymph and blood to the body's storage depots. The liver, which has a large capacity for storage, contains about 90% of the vitamin A found in the body.

Isolation and Synthesis. It was not until 1937 that pure, crystalline vitamin A was isolated by Holmes and Corbett from fish-liver oil. Research in the synthesis of vitamin A was stimulated during the Second World War by the threat to natural sources in fish-liver oil. This work resulted in several processes to produce vitamin A synthetically. Of these, one of the most successful was evolved by the Roche research team headed by Dr. Otto Isler which was announced in 1947. Much of the vitamin A now used for pharmaceutical manufacturing and food fortification in the United States and many other countries of the world is produced by this process.

Vitamin A and beta carotene produced by the Hoffmann-La Roche synthesis have many positive advantages for food and pharmaceutical manufacturers. Among these are the complete absence of any "fishy" odor, taste, or after-taste; the reasonable and stable price; their stability, purity and uniformity and the assurance of a steady economical supply.

Vitamin A is described in the U. S. Pharmacopoeia as a fully acceptable compound for use in pharmaceutical products. The U. S. Food and Drug Administration has approved its use for the fortification of margarine and other foods.

Human Requirements. The Food & Nutrition Board of the National Research Council recommends that a daily allowance of 5000 International Units of vitamin A in the diet will maintain good nutrition of healthy adults in the U.S.A. The Board's recommendations vary above this for pregnant and lactating women, and below for infants and children. The adult dose used in therapy may vary from 50,000 to 200,000 units daily.

Production. At the huge Roche Park plant of Hoffmann-La Roche in Nutley, New Jersey, over 500 pieces of large-scale chemical engineering equipment are working constantly to produce the superior 'Roche' vitamin A. This production is so large that 'Roche' vitamin A comes from the plant actually by the tons. In fact, the entire needs of the food and pharmaceutical industries of the United States and Canada can be met from this one plant.

Again the Roche people, who have so often led the way in vitamin research and production, have made another contribution to the better health and well-being of countless millions.

This article is one of a series giving a brief resume of our present knowledge of those essential health factors —the vitamins. Reprints of this and other articles are available without charge. The Vital Story of Vitamin C was published previously and others are forthcoming. Send your request to: Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul St., W.; Montreal, Quebec.

QUANTITATIVE ASPECTS OF STRAWBERRY PIGMENT DEGRADATION[a, b]

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(Manuscript received June 13, 1956)

Previous investigations, including those of Kertesz and Sondheimer *(8)*, Mackinney and Chichester *(13)*, Meschter *(14)*, and Nebesky *et al. (15)*, have established that the red pigment of strawberry products is unstable in heating and storage, and that many factors can affect the rate at which this pigment degrades. In a recent contribution from this laboratory *(4)*, the changes in color and pigment content of strawberry jellies were studied in relation to the amount of heat applied in processing and in storage.

Much of the research carried out by the aforementioned investigators dealt with reactions of strawberry pigment in juice and/or partially purified pigment preparations. A notable exception was the investigation of Sondheimer and Kertesz *(20* and *21)* into the interrelationship of pigment, ascorbic acid, oxygen, and hydrogen peroxide.

In the present research, the degradation of the anthocyanin primarily responsible for the red color of strawberries was studied in pure pigment solutions. The effects of several factors (temperature, time, pH, oxygen, ascorbic acid, 5-hydroxymethyl-2-furaldehyde, and various potential stabilizers) on the rate of the degradation of this pigment were investigated quantitatively. An effort was also made to evaluate results obtained from the pure pigment study in terms of stabilization of the red color of strawberry juice.

EXPERIMENTAL

Preparation of pure strawberry pigment. Chromatographic analysis reveals the presence of two red pigments in strawberry juice *(14)*. One of them is present in much greater quantity than the other, and has been identified as pelargonidin 3-monoglucoside *(19)*. In the early stage of the present investigation, the picration method *(19)* was used for the preparation of the major pigment. Later, it was found that paper chromatography results in a greater yield of major pigment and at the same time separates the minor pigment, recently identified as cyanidin-3-glucoside by Lukton, Chichester, and Mackinney *(12)*, in recoverable quantities. The paper chromatographic technique used consisted of extracting strawberry juice with one-butanol, shaking the butanolic extract with 3 volumes of hexane, and streaking the red lower phase, which results from the previous step, on Whatman No. 1 paper. Triangular strips of paper (8 in. base x 20 in. height) were used, with the mixture to be resolved applied as a narrow streak 1 inch from the base and parallel to it. The solvent was a mixture of

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[a] Presented at the Sixteenth Annual Meeting of the IFT at St. Louis, June 13, 1956.

[b] Contribution No. 1052, Massachusetts Agricultural Experiment Station. This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 663 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsements of the Department of Defense.

one-butanol:acetic acid:water (40:10:25 v/v). This is a one-phase mixture. After 16 to 20 hours of ascending development at room temperature, the triangles were dried, and the colored zones cut off and eluted, using methanol containing a trace of HCl. The methanolic eluates were evaporated *in vacuo*, leaving an amorphous residue of each of the two pigments. In order to obtain the major pigment in crystalline form, the residue corresponding to this pigment was dissolved in water and rechromatographed as before. The methanolic eluate of the second run was acidified to contain 5% HCl and allowed to evaporate slowly. Dark-red needles were obtained whose spectrum was identical with that reported by Sondheimer and Kertesz (*19*) for pelargonidin 3-monoglucoside.

Kinetics of the pigment degradation. Crystalline pelargonidin 3-monoglucoside was dissolved in citrate-HCl buffer (0.05 M disodium citrate), at pH 2.0 and 3.4. The concentration of the pigment was approximately 0.1 mM. About 3 ml. of pigment solution was placed in Pyrex tubes 15x125 mm. The tubes were sealed, using an oxygen torch, and immersed into a constant temperature oil bath. Five temperatures were used: 45° C., 60° C., 80° C., 100° C., and 110° C. For the lower temperature, an air oven was employed. At suitable intervals of time, two tubes were taken out of the constant temperature bath at random, cooled in ice-water, and their pigment content was determined colorimetrically, using a reference curve prepared with crystalline pigment. A Beckman Model DU Spectrophotometer[c] was used. Measurement was made at wavelength of 500 mμ using a slit-width of 0.05 mm. It was not necessary to adjust the pH of the solution at two different values in order to estimate the pigment content, as customarily required by the method of Sondheimer and Kertesz (*18*), since no interference occurred in these model systems. When the effect of anaerobic conditions was studied, the tubes containing the solution were drawn to an open capillary and placed horizontally in a vacuum desiccator. The pressure was reduced to 3 cm. of Hg, and nitrogen, bubbled through alkaline pyrogallol (25% pyrogallol, 6% NaOH), was introduced into the system. The evacuation and nitrogen substitution was repeated five times.

A 24-gauge copper-constantan thermocouple was used with a potentiometer[d] to calculate, by Ball's (*1*) method, the lag-time correction for heating the samples in the oil bath.

Spectral changes of the pigment during its degradation. Distilled water solutions of the crystalline major pigment (0.15 mM) were prepared, and the pH was adjusted to three levels, 2.02, 3.52, and 5.38, using HCl. To a fourth solution of the same pigment concentration, ascorbic acid was added (3 mM final concentration), and the pH was adjusted to 3.42. The absorption spectra of these solutions were determined by means of a Beckman Model DU Spectrophotometer and 1-cm. silica cells. Two ml. of each solution were diluted to 10 ml. with citrate—HCl, pH 2.00, and the pH of the diluted solution was checked to insure that it was 2.00. The light transmittance was measured after allowing the dilutions to stand at room temperature for one hour. Optical density measurements were made in the range from 220 to 600 mμ immediately after preparation of the solutions, again after heating 3-ml. aliquots in flame-sealed Pyrex tubes at 100° C. for 20 minutes, and a third time after storing these heated solutions at 35° C. for 7 days. The solution at pH 3.52 was examined a fourth time after it became virtually colorless (about 5 weeks at 35° C.) Since a fine precipitate could be seen in this last solution, the supernatant liquid only was examined.

Oxygen, ascorbic acid and pigment. In the study of the kinetics of the pigment degradation, it became apparent that replacing the air by nitrogen in the tube containing the solution resulted in higher pigment retention. This was attributed to the detrimental effect of the oxygen on the pigment. In a further experiment, the role of the oxygen was therefore investigated in conjunction with the effect of ascorbic acid on the pigment. Six model systems, coded as follows, were prepared and followed for

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[c] Manufactured by Beckman Instruments, Inc., South Pasadena, Calif.

[d] Manufactured by Leeds and Northrup Co., Philadelphia, Pa.

changes in pigment (P) and in ascorbic acid (A) content, in the presence (+) and in the absence of each other and of oxygen (O): P, A, P + A, P + O, A + O, P + A + O. The systems contained a 0.15 mM concentration of the pigment, and a 5 mM concentration of ascorbic acid in 0.05 M citrate buffer at pH 3.4. The air was replaced by nitrogen, using the technique already described. The methods of Sondheimer and Kertesz (*18*) and Robinson and Stotz (*17*) were used for the determination of the pigment and the vitamin, respectively. After sealing, the tubes (Pyrex, 15 x 125 mm.) containing the solutions were immersed in an oil bath at 60° C. and determinations were performed after 0, 15, 30, 45, and 63 hours.

Oxygen, ascorbic acid oxidase and pigment in strawberry juice. In view of the detrimental effect of oxygen and ascorbic acid on the pigment as shown in the present study on model systems and elsewhere, (*15* and *21*), the removal of ascorbic acid and headspace and dissolved oxygen were studied as possible means of extending the life-span of the pigment in strawberry juice. The ascorbic acid was removed by treating the juice with ascorbic acid oxidase[e] (250 units per 100 g. of juice). Prior to the addition of the enzyme, the pH of the juice was raised to 5.3, using 20% NaOH. Within an hour after the addition of the enzyme, at 25° C., the ascorbic acid of the juice was reduced from 60 mg. % to 3 mg. %, after which the pH was restored to its original level of 3.45 by means of a 30% citric acid solution. Half of the enzyme-treated juice was tubed and sealed under air left in; whereas, the other half was sealed under a nitrogen headspace. Similarly, enzyme-untreated control juice was tubed and sealed in the presence or absence of air. The 4 groups of tubes were immersed in an oil bath at 60° C., and pigment determinations were carried out after 0, 20, 40, and 60 hours of exposure.

5-Hydroxymethyl-2-furaldehyde and pigment. It is a characteristic reaction of hexoses to form 5-hydroxymethyl-2-furaldehyde (HMF) upon heating with acids in solution. Since hexoses are present in strawberries, and added or formed from sucrose in the preparation of strawberry spreads, the effect of this very active aldehyde on the pigment was investigated. Crystalline HMF, prepared by the method of Haworth and Jones (*6*) was added at a 20-mM concentration to 0.05 M citrate buffer, pH 3.4, containing 0.1 mM of pure pelargonidin-3-monoglucoside. A blank, with no added HMF, was also used. Three-ml. samples were transferred into 15 x 125 mm. Pyrex tubes, which were then flame-sealed, heated for 5 minutes at 100° C., and stored at 40° C. Pigment determinations were made before the tubes were sealed, and after 2, 4, 8, 16, and 25 days, using Sondheimer and Kertesz's (*18*) method.

Chemical additives. As a practical approach to the problem of fixing the natural red color of strawberry products, various chemical substances were added to strawberry juice, and to 0.05 M citrate buffer solutions containing 0.01 mM of pelargonidin-3-monoglucoside. Most of the additives used were selected on the basis of their high or low oxidation-reduction potential, or of their metal-chelating properties.

The juice and the buffered pigment solutions had the same pH of 3.42. Three-ml. samples were filled into 15 x 125 mm. test tubes. Sealed tubes were heated for 5 minutes at 105° C., and stored at 35° C. Before heating, and after 12 days of storage, the samples were suitably diluted with citrate buffer at pH 3.42 and the optical density of the dilutions was determined at 500 mμ at one pH, using a Beckman Model DU Spectrophotometer. Color retention was derived from transmittance data calculated on the assumption that Beer's law was obeyed in all cases.

The effects of phytic acid and of $AlCl_3$ were investigated in a separate experiment. Phytic acid has been reported to have prevented fading of the red color of sour cherries (*3*). Aluminum was tried because of the possibility of forming, by means of its electronic sextet, an addition product with the oxygen of the pyrylium ring, thereby stabilizing the pigment molecule (*9*). Strawberry juice was used, and the phytic acid[f]

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[e] Obtained from Nutritional Biochemical Corp., Cleveland 28, Ohio.

[f] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

and $AlCl_3$ were added at the levels of 0.5 and 1.0%. The method of Sondheimer and Kertesz (*18*) was used for the determination of the pigment.

RESULTS AND DISCUSSION

The kinetics, the spectral changes, and the mechanism of the pigment degradation. When air was left in the buffered pigment solution, the thermal destruction rate curves of the pigment were not straight lines when the log of the retention was plotted against time (solid lines in Figure 1). When, however, the air was replaced by nitrogen, straight-line destruction

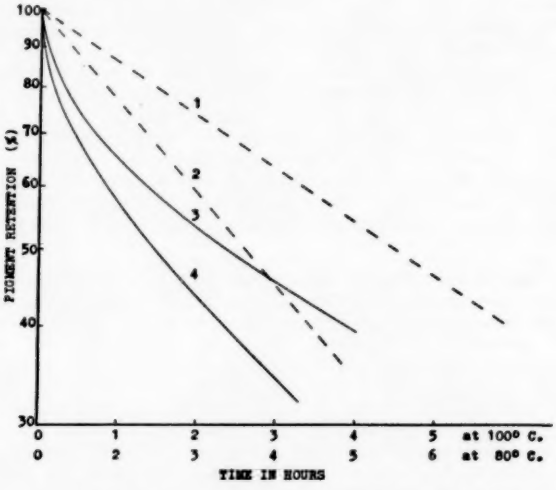


Figure 1. Retention of pelargonidin-3-monoglucoside in citrate buffer solutions. Legend:

1. **pH 3.4; stored at 80° C. under nitrogen**
2. **pH 2.0; stored at 100° C. under nitrogen**
3. **pH 3.4; stored at 80° C. under air**
4. **pH 2.0; stored at 100° C. under air**

curves were obtained. The dotted lines in Figure 1 and solid lines in Figure 2 are based upon such data. It was apparent, from the results obtained, that in the absence of oxygen and in the temperature range of 45° C. to 110° C., the destruction of the pigment is a first-order reaction, at least at pH 2.0 and 3.4. Since no destruction of the pigment was noticed when the pigment was stored dry, in crystalline form or on the chromatograms, water appears to be involved in the above decolorization reactions. Hydrolysis of the glycosidic bond would be a possibility, since such a reaction would liberate an aglycone which decolorizes much faster than the glycoside (*7*). However, extraction of the pure pigment solutions with ethyl acetate, at several stages during the decolorization process, failed to detect the presence of the aglycone. Another type of hydrolysis was therefore considered, involving opening of the pyrylium ring at position

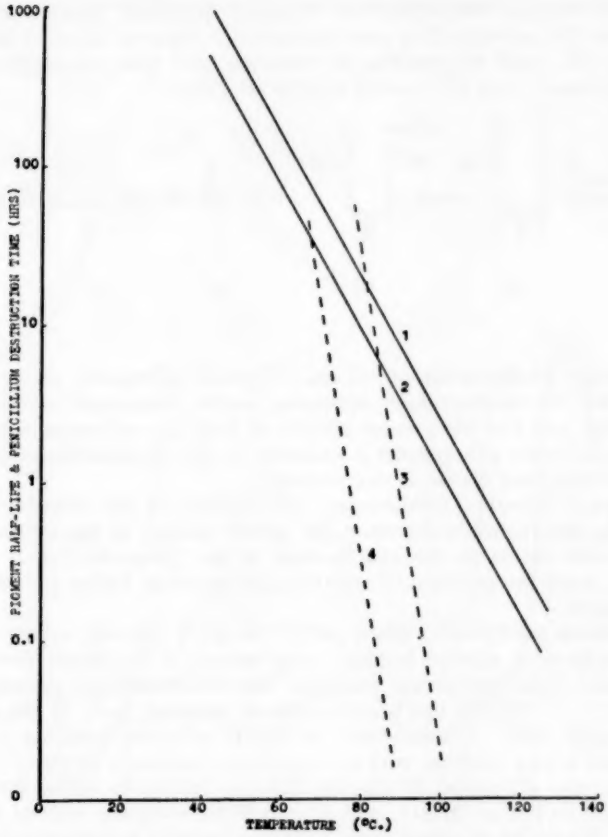
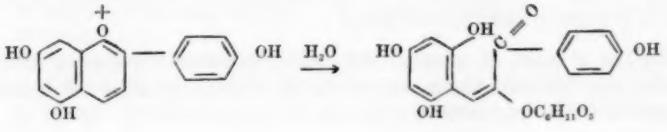


Figure 2. Thermal destruction-times of pelargonidin-3-monoglucoside in buffered solution, and a Penicillium in diluted blueberry juice.

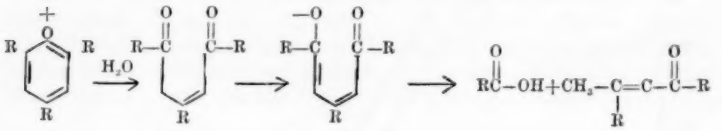
Legend:

1. **Pigment, pH 2.0**
2. **Pigment, pH 3.4**
3. **Sclerotia of Penicillium**
4. **Ascopores of Penicillium**

1-2, with formation of a ketone, i.e., a substituted chalcone. Further degradation of this ketone would eventually lead to the brown precipitate, which has constantly been observed as an end-product of the degradation of the pigment.



Berson *(2)* showed that crystalline triphenyl pyrylium pseudobases, obtained from the corresponding oxonium salts (I), occur in an open diketone form only (II), and on standing or warming fade with concomitant formation of benzoic acid (IV) and a neutral oil (V).



I II III IV V

R=C_6H_5

The maximum of absorption at 247 mμ of Berson's diketones, which is very close to the 250 mμ maximum appearing in the strawberry anthocyanin degradation, and also the greater lability of both the anthocyanin and the diketones at higher pH, suggest a similarity in the degradation mechanism of the two pyrylium classes of compounds.

Figures 3 indicates, furthermore, that heating of the strawberry pigment in aqueous solution decreases the optical density of the solution to a much greater extent in the visible than in the ultraviolet region of the spectrum, while the addition of ascorbic acid or use of higher pH enhances these changes.

The brown precipitate. When any of the pure pigment solutions used in this study were allowed to stand long enough, a red-brown precipitate settled out. This precipitate displayed the characteristics described by Pederson et al. *(16)* for the brown sediment obtained early in the storage of strawberry juice. It was soluble in NaOH solutions, yielding a yellow rather than brown solution, with an absorption maximum at about 420 mμ.

It was also attempted to dissolve this precipitate in ether, petroleum ether, conc. HCl, conc. H_2SO_4, and 5% $NaHCO_3$ solutions without success. However, methanol dissolved part of it, the residue being soluble in 5% NaOH solution.

In an experiment in which pelargonidin-3-monoglucoside-C^{14} was stored in citrate buffer solution, pH 3.4, until virtual decolorization, 85% of the original radioactivity of the pigment was recovered in the brown precipitate *(11)*.

The brown end-product of the strawberry anthocyanin degradation must contribute significantly to the darkening of strawberry spreads in storage. Studies with model systems not containing anthocyanin have shown a much less marked browning than is necessary to account for the darkening of strawberry spreads *(10, 22)*.

Oxygen, ascorbic acid, and pigment. The results of the study on the effect of oxygen and ascorbic acid on the pigment in model systems are shown in Figure 4 and indicate that:

1. In the absence of oxygen, the major strawberry pigment alone, or the ascorbic acid alone, are relatively stable, the pigment being less stable than the vitamin.

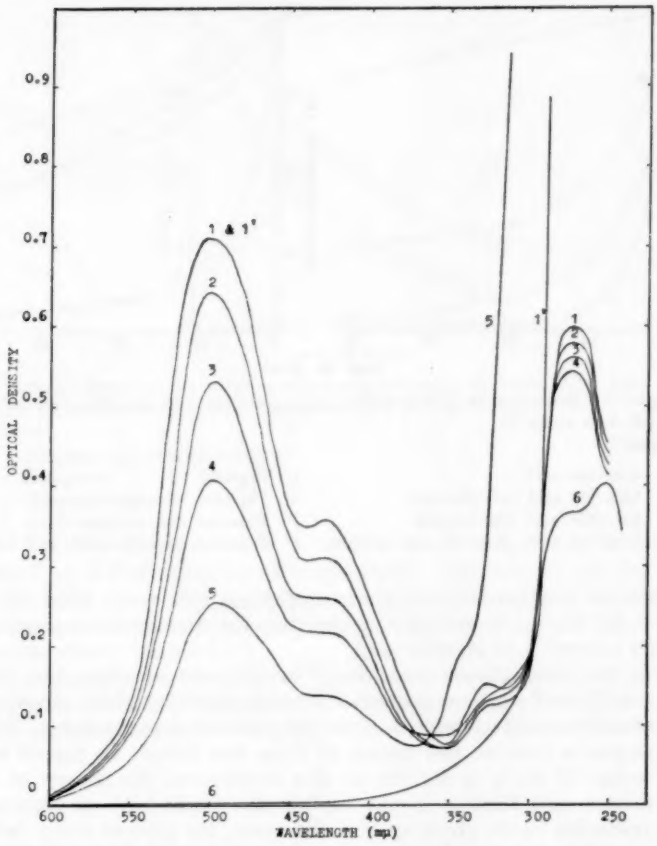


Figure 3. Effect of heating, storage, pH and ascorbic acid on the absorption spectra of aqueous 0.15 mM pelargonidin-3-monglucoside solutions. (All spectral measurements carried out at pH 2.0)

Legend:

1. **Before heating**
1′ **Before heating, 3 mM ascorbic acid added**
2. **After heating at pH 2.02 (20 minutes at 100°C.)**
3. **After heating at pH 3.52**
4. **After heating at pH 5.38**
5. **After heating at pH 3.42, 3 mM ascorbic acid added**
6. **Heated at pH 3.52 and stored for 5 weeks at 35° C.**

2. In the presence of oxygen, the pigment alone, or the ascorbic acid alone, are relatively unstable, the pigment being more stable than the vitamin.
3. In the absence of oxygen, the simultaneous presence of pigment and ascorbic acid leads to a faster degradation of both than when each is alone. Stoichiometric relationships, however, do not suggest a

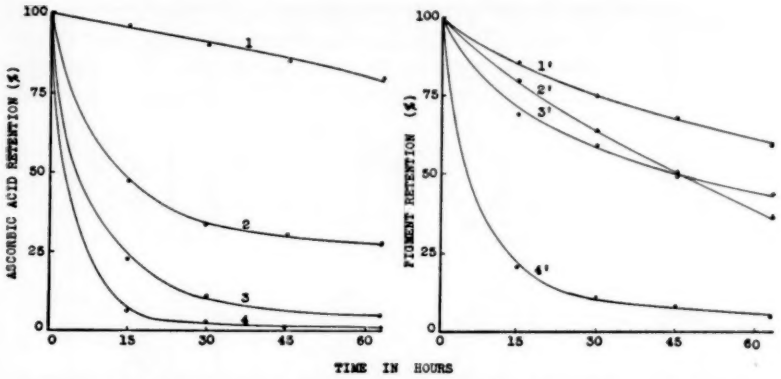


Figure 4. Retention of pelargonidin-3-monoglucoside and ascorbic acid in citrate buffer, pH 3.40 at 60° C.

Legend:

1. Ascorbic acid	**1′. Pigment**
2. Ascorbic acid and pigment	**2′. Pigment and ascorbic acid**
3. Ascorbic acid and oxygen	**3′. Pigment and oxygen**
4. Ascorbic acid, pigment, and oxygen	**4′. Pigment, ascorbic acid, and oxygen**

simple reaction between pigment and ascorbic acid. After 15 hours at 60° C., e.g., 1 molecule of pigment was destroyed for about every 50 molecules of ascorbic acid.

4. In the simultaneous presence of oxygen and ascorbic acid, the destruction of pigment is greater than the addition of the single effects of oxygen and ascorbic acid on the pigment accounted for. This indicates a positive interaction of these two factors in regard to pigment. If H_2O_2 is formed in this interaction, the theory of Sondheimer and Kertesz would explain the rapid rate of pigment degradation in the above system. However, the present study indicates that there is more than one path for the degradation of the pigment to follow in such a system.

The above model system findings are corroborated by other data on the retention of pigment in strawberry juice stored in the presence of oxygen. In one experiment, ten times as much pigment (30% retention) was found in untreated strawberry juice stored at 50° C. for 160 hours under nitrogen, than in the same juice stored under air (3% retention). These results are in general agreement with the earlier findings of Sondheimer and Kertesz (*21*).

The results of experiments with ascorbic acid oxidase (Figure 5) indicate that it is possible to increase slightly the pigment retention in strawberry juice by destroying the ascorbic acid enzymatically. It is, however, obvious that the beneficial effect of the enzyme treatment is not nearly as great as that of oxygen exclusion. A combination of ascorbic acid oxidase treatment and nitrogen headspace resulted, of course, in the highest level of pigment retention.

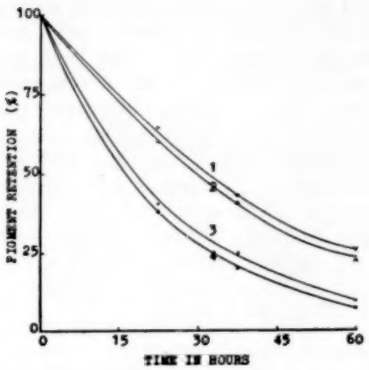


Figure 5. Retention of pelargonidin-3-monoglucoside in strawberry juice treated with ascorbic acid oxidase during storage at 60°C.

Legend:

1. **Enzyme treated and deaerated**
2. **Deaerated**
3. **Enzyme treated**
4. **Control**

Effect of 5-hydroxymethyl-2-furaldehyde. The results of the HMF experiment are shown in Figure 6 and corroborate Meschter's findings (*14*) that the products formed on heating acidic solutions of sugars are harmful to the strawberry pigment.

Effect of additives. Out of the 19 different additives tested as potential stabilizers in this study, only thiourea showed a marked stabilizing effect on both strawberry juice and a buffered pigment solution. Thiourea had

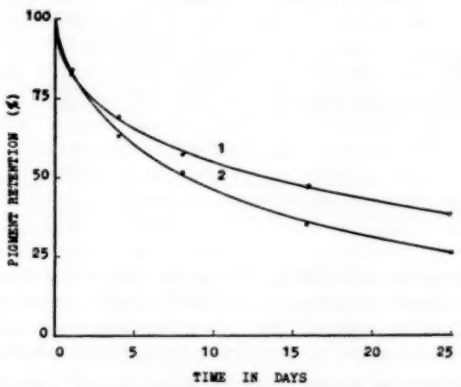


Figure 6. Effect of 5-hydroxymethyl-2-furaldehyde on the retention of pelargonidin-3-monoglucoside in citrate buffer at pH 3.40, during storage at 40°C.

Legend:

1. 0.1 mM pigment
2. 0.1 mM pigment and 20 mM HMF

been previously found by other investigators to decrease the rate of pigment destruction in the presence of ascorbic acid and air *(21)*. Studies conducted in this laboratory have shown that heating thiourea with citric acid and sugar in solution results in the development of a pronounced red-brown color. Formation of colored end-products absorbing at 500 mμ must be the reason for the increase in optical density above the original value on heating and storing strawberry juice with added thiourea. Thiourea is toxic and could hardly be used as a food additive; it might, however, serve as a prototype in the search for other stabilizers.

Propyl gallate and quercetin showed a slight protective effect on the color of both juice and pigment solution. Phytic acid (0.5 and 1.0%) and $AlCl_3$ (0.5 and 1.0%) addition caused no increased retention in strawberry juice stored at 35° C. for 3 weeks.

TABLE 1

Effect of additives on the optical density of strawberry juice and pelargonidin-3-monoglucoside in citrate buffer stored at 36° C. for 12 days[1]

Additives	Concentration (%)	Optical Density (at 500 mμ)	
		Juice	Pigment
Control—before storage	—	.820	.545
Control—after storage	—	.560	.414
dl—alanine	0.1	.533	.395
Ascorbic acid	0.2	.323	.023
Calcium pantothenate	0.002	.575	.412
Cystine	0.0001	.675	.400
Gallic acid	0.2	.682	.408
Kojic acid	0.2	.522	.354
Phosphates[2]	0.67	.570	.380
Propyl gallate	0.2	.600	.451
Quercetin	0.1	.702	.465
Riboflavin	0.0002	.670	.322
Rutin	0.1	.595	.405
Sequestrene	0.002	.535	.416
Stannous chloride	0.002	.568	.415
Stannic chloride	0.002	.530	.410
Stannous chloride & sequestrene	0.002	.495	.495
Tannic acid	0.2	.725	.391
Thiamine	0.0002	.552	.429[3]
Thiourea	0.2	1.080	.470

[1] Samples heated for 5 minutes at 105° C. prior to storage.
[2] 20 g. Na_3PO_4 and 20 g. Na_2HPO_4, dissolved in 100 cc. H_2O (5).
[3] Six days' storage.

Food processing considerations. The data obtained in the present study of strawberry pigment stability, in conjunction with information published by other investigators, suggest that the following factors should be considered in the manufacture of preserved strawberry products:

1. Use of the lowest possible temperature on all stages of processing, packaging, and storage.
2. Removal of the oxygen from the product.
3. Selection of strawberry varieties of high pigment content and low ascorbic content.

4. Decrease of the pH within acceptable limits, preferably by using citric acid.
5. Avoidance of contamination with metallic ions.

Since heat is as yet still considered to be the only practical means of preserving fruit spreads, is it advantageous from the standpoint of strawberry pigment retention to use low temperature—long time heat treatments, or high temperature—short time treatments?

In evaluating heat processes of food, the most heat-resistant microorganism, the one likely to be a health hazard or to cause food spoilage, is conventionally used as a test organism. The unidentified species of *Penicillium*, which was isolated from canned blueberries by Williams, Cameron, and Williams *(23)*, is very heat-resistant in acid media and is capable of growing under high vacuum. Its thermal death-time characteristics have been studied in diluted blueberry juice and reported by the above-mentioned authors. As data on the thermal resistance of molds in preserve-type media are not readily available, the blueberry organism will be used in estimating lethality in the present discussion, bearing in mind the difference in pH and solids content between diluted blueberry juice and strawberry spreads.

Figure 2 shows, along with the thermal destruction-time curves (1 and 2) of the major strawberry anthocyanin, the thermal death-time curves (3 and 4) for the sclerotia and ascopores of the test *Penicillium*, respectively.[8] A simple inspection of the curve indicates that high-short heat treatments will cause less pigment destruction while exterminating the mold than low-long ones, since the organism is more sensitive than the pigment to increases in temperature.

A quantitative interpretation on the interrelationship of the 4 factors: i.e., time-temperature-death of the organism-pigment destruction, is illustrated in Figure 7, in which the retention of pigment is plotted against the temperature of microbially equivalent heat treatments. The two curves of Figure 7 were obtained by taking from the dotted lines 3 or 4 of Figure 2 the temperature required to exterminate the sclerotia or ascospores at various times, and securing the per cent destruction of pigment for each such time-temperature pair at the buffer solution of pH 3.40 (which is close to the pH at which the mold death curves were obtained). It is apparent that a heat treatment at 100° C., at which a minute or two would exterminate the sclerotia, would leave the pigment practically undamaged; whereas, heating at lower temperatures would necessitate times capable of causing considerable pigment destruction. Similarly, if the ascospores were to be exterminated only, heating at 80° C. for 20 minutes would kill all these cells and cause very little damage to the pigment; while at lower temperatures, times of exposure would be necessary which would result in an appreciable loss of pigment.

It is not intended that the figures cited above be taken as heat processing specifications for good pigment retention in strawberry products.

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[8] Curves 3 and 4 were obtained from the paper by Williams, Cameron, and Williams *(23)*.

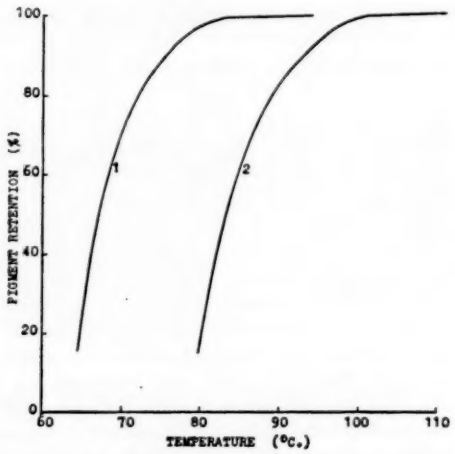

Figure 7. Retention of pelargonidin-3-monoglucoside subjected to minimum heat treatments, at various temperatures, required to destroy the sclerotia and ascopores of Penicillium mold.

1. **Ascopores**
2. **Sclerotia**

For such a purpose, the destruction of the pigment and of the most heat-resistant undesirable microorganism should be studied in the very product to be processed. Meschter *(14)* gives a heat destruction-time curve for the pigment in strawberry preserves with a z value, which appears slightly higher than the one obtained with the model systems in this study. This further emphasizes the value of high-short treatment in the commercial practice, when heat is used for preservation only, and against organisms of small z value.

SUMMARY AND CONCLUSIONS

1. The kinetics of the degradation of pelargonidin-3-monoglucoside were studied in buffer solutions, at pH 2.0 and 3.4. Under exclusion of oxygen and in the temperature range from 45° C. (113° F.) to 110° C. (230° F.) first-order reaction rates and straight thermal destruction-time lines were obtained.
2. A tentative scheme for this pigment's degradation was proposed. This scheme involves hydrolytic opening of the pyrylium ring, with formation of a substituted chalcone, further degrading to a brown, insoluble polyphenolic compound.
3. In studying the interaction of oxygen, ascorbic acid, and pigment in model systems, oxygen or ascorbic acid was shown to be detrimental to the pigment. These two agents combined caused more destruction of pigment than the sum of their single effects. The pigment retention in strawberry juice stored under nitrogen was also several-fold higher than that in similar juice stored under air.

4. Destroying the ascorbic acid of strawberry juice by means of ascorbic acid oxidase resulted in greater pigment retention. However, when the enzyme treatment was combined with the removal of the oxygen in the juice, the resultant pigment retention was only slightly higher than that which was obtained through oxygen removal alone.
5. Assuming that heating is necessary to prevent only microbiological spoilage in a strawberry product containing an organism having the thermal death-time characteristics of the *Penicillium* described by Williams, Cameron, and Williams *(23)*, the highest level of pigment retention would be obtained by the use of a short time–high temperature treatment.
6. The effect of HMF on pigment destruction was studied in pure solutions. It was found that the destruction of pelargonidin-3-monoglucoside is accelerated by this sugar-degradation product.
7. The effects of 19 different additives were tested on color retention in strawberry juice and buffered pigment solutions. Only thiourea, propyl gallate, and quercetin showed any beneficial effects on both juice and pigment.

Acknowledgments

The authors are indebted to Dr. M. A. Steinberg for the synthesis of the hydroxymethylfurfural used in these studies and data on thiourea-sugar-acid reactions, and to Dr. W. B. Esselen for his guidance and assistance in the processing aspects of this research.

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NUTRIENT COMPOSITION OF CUBAN FOODS [a, b]
II. FOODS OF VEGETABLE ORIGIN

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A study of Cuban foods was undertaken early in 1952 by this laboratory. The first report of the results of this work was given by Navia *et al. (9, 10)*. This investigation has been continued along the same lines and a number of varieties of other foods have been analyzed, including 12 strains of corn and 14 strains of rice. Results from the analysis of 137 samples of food of vegetable origin, collected in various parts of Cuba between February and December (1954) are reported in the present paper. These represented 62 different foods, 4 of which were obtained in the market and 133 from the field. Many of these samples were obtained from regions not surveyed previously.

METHODS

Techniques used in the collection, preparation, and analysis of each sample for 16 nutrients were the same as those described in the first paper of this series *(9)*. Dry seeds were shipped in sealed jars without further preparation. Within a few hours of collection, the wet food materials were slurried in a Waring blender, stabilized, sealed in glass jars, then placed in refrigeration until shipped. All shipping boxes reached the laboratory within 12 hours.

Three slurries of each sample were prepared by a technique developed by Munsell *et al. (8)*. Subsample A was prepared by blending 100 g. of the edible portion with 100 ml. of alkaline alcohol solution (30 g. KOH, 320 g. 95% ethyl alcohol, and water to make 1000 ml.). Subsample B was prepared by slurrying 400 g. of sample with 200 to 400 ml. of 1% oxalic acid solution. Just before the blending was completed, 10 and 5 ml. of chloroform were added to subsamples B and C, respectively. Sub-

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sample A was used for carotene analysis, subsample C for ascorbic acid analysis, and subsample B for all the remaining analyses.

Moisture, ether extract, crude fiber, ash, and calcium were measured according to methods described in the A.O.A.C. *(1)*, nitrogen by the method of Hamilton and Simpson *(4)*, iron by the α-dipyridyl procedure *(3)*, phosphorus by the modification suggested by Lowry and Lopez *(5)*, and carotene by the Wall and Kelley modification *(16)* of the method of Moore *(7)*. Ascorbic acid was determined always within 48 hours after arrival at the laboratory by the Roe and Oesterling method *(11)*. Thiamine and riboflavin were estimated fluorimetrically *(8)*, niacin microbiologically (*L. plantarum* 17-5) *(15)*. Tryptophan was extracted by the Greene-Black *(2)* and the Schweigert-Snell *(12)* procedures and estimated microbiologically (*L. plantarum* 17-5). Methionine and lysine were hydrolyzed from the food proteins *(12, 13)* and analyzed microbiologically according to Steele *et al. (14)* using *Leuconostoc mesenteroides* P-60 as test organism. In the methionine assay the amino acids in the culture medium were replaced by hydrogen peroxide-treated peptone, with the addition of cysteine, tyrosine, and tryptophan *(6)*.

DESCRIPTIONS AND USES OF FOODS STUDIED

Cereals

Corn: (Zea mays). Twelve samples of corn (maiz) were collected for this study. One was in the milk stage and the other 11 were dry samples. They represented the most common types of corn cultivated in Cuba.

Rice: (Oryza sativa). The rice (arroz) samples collected can be grouped into:

A) *Rice, brown:* One sample of brown rice (arroz integral) belonging to the Bluebonnet type.

B) *Rice, polishings:* The 3 samples (a, b, c) of rice polishings (polvo de arroz) represented the polishings obtained from the first, second, and third millings, respectively.

C) *Rice, polished:* Seven samples of polished rise (arroz pulido) were collected. Sample (a) belonged to the short grain type called "bolo," and samples (b, c, d, f, g) to the most common long grain types, mainly Century, Bluebonnet, and Rexora.

D) *Rice, unpolished:* Three samples of unpolished rice (arroz no pulido) were obtained. Sample (a) was processed by hand on a wooden mill in the most primitive way. The other two samples (b and c) were milled by modern methods. All 3 samples were harvested in 1952.

Earth Vegetables

Arracacha: (Arracacia xanthorrhiza). The arracacha (afio) used in this study was obtained in the municipal market in Guantanamo. It came from a region where the altitude is more than 2,000 feet. The sample consisted of 8 units weighing 1,300 g.

Carrot: (Daucus carota). The carrot (zanahoria) samples consisted of mature roots.

Fruit Vegetables

Pepper: (Capsicum frutescens). The common bell pepper (aji) was collected. Sample (c) was green and (d) was ripe. Two horticultural varieties not previously studied are included: "cachucha" (a) a dorsi-ventrally compressed, small-ribbed pepper; and "pimiento de conserva" or "pimiento Morron" (b), the thick-fleshed paprika type used in preserved form for flavoring and decorating such dishes as chicken-and-rice. The seed of the latter had been imported from the Canary Islands. The stems, cores, and seed were discarded in preparing the sample.

Plantain: (Musa paradisiaca). Nine plantain (platano) samples were collected. Samples (a, b, c, d) correspond to the so-called "burro" type, a pale-fleshed plantain, and samples (e, f, g, h, i) to the "macho" type. They are eaten at both the green and ripe stage. Samples (a, b, e, f, g) were green, and samples (c, d, h, i) were ripe.

Squash: (Cucurbita moschata). The samples of squashes *(calabaza)*, used for this study were winter squashes of 3 degrees of ripeness: sample (a) was ripe, (b) was intermediate, and (c) was slightly green. Each was obtained from a different province in the Island.

Herbage Vegetables

Chive: (Allium schoenoprasum). The chive (cebolla de la tierra) is a common cultigen of Europe and Asia, grown in Cuba mainly by Chinese for their own use. The green hollow awl-shaped leaves are used for their mild onion flavor. Only the roots were discarded from the sample.

Leek: (*Allium porrum*). The leek (ajo puerro) is native to the eastern Mediterranean and has been in cultivation since very early times. It is not common in Cuba, probably because it is not fully adapted to this climate. The thick, flat, broad leaves are cooked as a green vegetable or used in soup. The wilted leaf tips and the roots were discarded.

Ramie: (Boehmeria nivea). The ramie plant (ramie), until very recent years used only as a source of the fiber of the same name, was found during studies in Central America to have a high food value. The present sample was prepared entirely from the top 4 inches of the leafy tops of stalks which were one to 2 feet tall and growing from an old planting at the Atkins Garden. The plant is a perennial herbaceous shrub native to Eastern Asia and is widely cultivated from Japan to India as a source of fiber.

Legumes and Oleaginous Seeds

Peanut: (Arachis hypogaea). The peanut (mani) and the seeds were shelled by hand. One sample (a) was slurried without removing the thin red skin. The seeds of another sample (b) were skinned and roasted, and those of a third (c) were toasted without removing the skin. All were of the same collection.

Fruits, Citrus

Lime: (Citrus aurantifolia). The lime (limon) is native to the East Indies but is not widely planted throughout the tropics and sub-tropics. It occasionally escapes from cultivation in Cuba and appears to be almost native. Three main groups are found here: "limon criollo" (c, d, e, g) which is the West Indian, Mexican, or Key lime, a small-fruited acid type; "limon persica" (f, h) the Persian or Tahiti Lime, a large-fruited and generally less acid type; and the "lima" (a, b), one of the Sweet Lime group, unidentified as to species *(1)*. The strained juice of the first two and the deseeded pulp and juice of the last were used for the samples.

Orange, sour: (Citrus aurantium). The sour or Seville orange (naranja agria) (a) is the orange which was first grown in western civilization, the sweet orange not having reached Europe until shortly before the discovery of America. It is intensely sour, with a bitter after-taste and is widely used for marmalade and, in Cuba for flavoring meats, especially roast suckling pig, steaks, and soups. Native to Southeast Asia, it is now cultivated throughout the tropics and sub-tropics. The sample was prepared from the strained juice. The orange, sour, (naranja cajel) (b) is one of the Bittersweet group of this species.

Fruits, Various

Antidesma: (Antidesma platyphyllum). The antidesma (antidesma) is a little known species of small tree from Hawaii, related to the Buni-berry (Antidesma bunius). The sample was collected from a plant growing in the Atkins Garden and was prepared by squeezing the juice through triple gauze. The small berries are borne in long densely packed racemes, and make an excellent jelly.

Barbados gooseberry: (Pereskia aculeata). The barbados gooseberry or lemon-vine (grosellero de la Florida) is also known in Cuba as "Corona de Noyia" and as "Guinda" although the latter name is properly applied to the Sour Cherry *(Prunus cerasus)* of Europe. It is a member of the cactus family, belonging to a section of the family which bears true leaves. It is shrubby at first and later becomes a vine. It is fairly common in Cuba, cultivated primarily for its fragrant and showy white, pale yellow or pink flowers, but also for its small yellow fruits which are used in cooling drinks and preserves. The sample was prepared from the juice of the whole fruits by squeezing through triple gauze.

Carambola: (Averrhoa carambola). The carambola (carambola) is a small tree originally found from China to India, and now cultivated elsewhere in the tropics.

It is known only in cultivation and appears to include several races, the common one of which bears very acid fruit suitable for pickles or tart salads, although another race bears sweet fruits of a plum-like flavor and texture. The small fruits, 3 to 5 inches long, are strongly 5 to 6 angled, and translucent amber in color when ripe. The tips and the seeds were discarded and the remainder used for the sample.

Cashew apple: (Anacardium occidentale). The cashew apple (marañon) is a small unattractive tree found on the northern Brasilian coast and the plains of the lower Amazon. It was brought to the West Indies before the time of Columbus, and is now widely cultivated throughout the lowland tropics. The stem of the fruit swells as the fruit matures, becoming, when ripe, a yellow or red apple-shaped pseudo-fruit, the cashew apple. The true fruit is a small green kidney-shaped terminal appendage which encloses the cashew-nut. The cashew apple sample was red-skinned and had a juicy yellow flesh. Only the juice was used for the sample. The best types have a pleasantly aromatic flesh and are good as dessert fruit. They may also be made into a wine which retains the aromatic quality. The trees are often found as escapes from cultivation or semiwild in Cuba, and are planted extensively for the nuts in some parts of Asia.

Custard apple: (Annona reticulata). The custard apple or Bullock's Heart (mamon) is also known in Cuba as "anon manteca" and, more commonly, as "chirimoya," although the latter name applies properly to the Chirimoya (Annona cherimola) of the tropical highlands, which does not fruit successfully in Cuba. It is a native of the Caribbean lowlands, and is cultivated in Cuba and throughout the Tropics. The tree bears heart-shaped reddish-brown fruits about 4 to 6 inches long which have a white, slightly granular, rather insipid pulp. The sample consisted of the pulp from fruits of better than average flavor and quality.

Garcinia, sweet: (Garcinia dulcis). The sweet garcinia (garcinia) is a native from Java to the Philippines and is cultivated in that region and rarely in other parts of the tropics. The peach-sized fruits are yellow, smooth-skinned and contain a bland somewhat tart, agreeably flavored pulp immediately around the seeds, which is best suited for making jam. This pulp was used in preparing the sample. The tree is an attractive ornamental and is being planted more for that purpose in Cuba than for its fruits; nevertheless it is still rare here.

Guava: (Psidium guajava). This sample was from a race of the common guava (guayaba del Peru) with pear-shaped fruits. Its association with Peru is not clear. The whole fruit was used to prepare the sample.

Honeyberry: (Melicoca bijuga). The honeyberry (mamoncillo) is also called the Spanish lime, although it is not a citrus fruit nor is it Spanish in origin. It commonly occurs as a cultivated or wild tree of the West Indies. It bears small, round, leathery skinned fruits which enclose a thin, jelly-like, acid pulp surrounding a large hard seed. The fruiting branches are sold in markets and by roving vendors, and the fruits are usually eaten fresh, although a sweet is prepared from them occasionally. The sample was prepared from the juice of the pulp which had been squeezed through a jelly bag.

Jaboticaba: (Myrciaria cauliflora). The Brasilian grape tree, also called jaboticaba in Spanish, is found both wild and cultivated in Southern Brasil. Its small fruits are the color and shape of Concord grapes. The thick skin encloses a deliciously flavored, white translucent pulp and a few small seeds. They are borne in clusters directly on the wood of the branches and stems, and appear several times a year. Excellent as fresh fruits, they also make a fine jelly or sauce, in which case the skin is left on to add color and tartness. The sample was prepared from the juice of the whole fruits squeezed through a jelly bag.

Mango: (Mangifera indica). The following races of mango (mango) were collected: "manga amarilla" (a), "manga blanca" (b), "mango mamey" (c), and "mango Toledo" (d).

Myrobalan: (Phyllanthus emblica). The myrobalan or emblic (mirobalanos) is a relatively rare shrubby tree introduced into Cuba from tropical Asia where it is both native and cultivated. The small, round fleshy fruits have a high tannin content, and were formerly used medicinally. Although there is no evidence of their use as food in Cuba, they are eaten as pickles, flavorings and sweets in southeast Asia. The fruits minus the seeds were slurried in preparing the sample.

Paniala: (Flacourtia catapharacta). The paniala (paniala) has been confused, both botanically and in common names, with the Governor's plum or Ramontchi *(Flacourtia indica)*, but is sufficiently distinct. It is a large, spiny shrub or small tree widely cultivated in Southeast Asia, but is rare in Cuba. Its small, tart, thin-skinned fruits are fairly good when eaten out-of-hand and are excellent as preserves. The strained pulp was used for the sample.

Sapodilla: (Achras zapota). The sapodilla (nispero) is native to Southern Mexico and Guatemala, where it is usually called "Chicozapote." In most parts of Cuba it is called "sapote," although this name is applied in other Spanish-speaking countries to the fruit which is called "mamey colorado" in Cuba. The true "nispero" is the medlar, a European plant not grown here. The tall tree bears round thin-skinned brown fruits with a translucent brown and sweet flesh which has been compared to a blend of pears and brown sugar. Commonly, this fruit is eaten out-of-hand, although it is occasionally made into a conserve. The latex tapped from the tree is the source of chicle, the basis for chewing gum. It is widely cultivated in the tropics for its fruit, which is one of the best. The flesh alone was used for the sample.

Sapote or Marmalade fruit: (Calocarpum sapota). The sapote or marmalade fruit (mamey colorado) is a native of the Central American lowlands and is now widely cultivated throughout Cuba, and much of the world tropics. The large tree bears ovoid fruits up to a foot long which have a brown scurfy skin, brickred flesh, and a single large seed. Since all trees are seedlings, the fruits are extremely variable in quality. The poorest taste like unripe squash, while the best make an excellent dessert fruit and a fine aromatic jam or marmalade. The samples were of ordinary quality only.

Star apple: (Chrysophyllum cainito). The star-apple (caimito) occurs in 2 forms: the "morado" with purple skin and flesh, and the "blanco" with green skin and pale tan flesh. The tall trees are handsome; the foliage is blue-green above and satiny-bronze beneath, and the fruits are best when eaten fresh, after they have been completely ripened on the tree. It is native to the West Indies and is common throughout Cuba. The seed-free flesh was used for the sample.

Tamarind: (Tamarindus indica). The tamarind, a large tree with dense attractive foliage is native to tropical Africa and Asia and is now cultivated throughout the tropics as an ornamental shade tree, as well as for its fruits. These thin-walled, slightly woody, brown pods are about 1 to 3 inches long and contain a few seeds enclosed in a sticky, fibrous dark brown pulp. The pulp, the part used for the sample, is both sweet and acid and makes an excellent cooling drink when beaten up in water. It is also used in making preserves. The pods and main fibers were peeled off and the pulp separated from the seed by rubbing through an aluminum mesh sieve.

Watermelon: (Citrullus vulgaris). The watermelon (melon de agua) was introduced in Cuba from its native tropical Africa, probably as one of the food crops for slaves. Grown with proper attention to its requirements, it produces melons of excellent quality here, although the average melon seen is second rate. The sample was prepared from the ripe flesh, the seed and rind being discarded.

Miscellaneous

Guava, processed: (product from Psidium guajava). Four samples of guava were collected. The guava marmalade (mermelada de guayaba) was obtained from the market and had been manufactured by an industrial concern. The guava pulp (pulpa de guayaba) sample consisted of guava shells, minus skin and seeds. It was comminuted and packaged in 5-gallon cans for use later in the preparation of marmalade. The 2 guava shells (cascos de guayaba) were taken from the same source: sample (a) was obtained before the process was finished, and sample (b) was prepared from the finished canned product.

Molasses, blackstrap: (product from Saccharum hybrids). The blackstrap or final molasses of commerce (miel final) is the molasses from which all the economically available crystallizable sugar has been removed by centrifugation. It is used as cattle feed and as the raw material for making industrial alcohol, rum and "aguardiente." The sample was of molasses from the 1954 grinding season.

RESULTS AND DISCUSSION

Results obtained in this study, summarized in Table 1, are expressed in terms of the content per 100 g. of raw, edible material. Certain of these samples were observed to contain significant amounts of some nutrients and samples of different strains of the same plant sometimes showed differences in composition.

Cereals

Corn: The 11 samples of dry corn studied varied considerably in content of iron, the values ranged from a low (2.54 mg%) for sample (d) to a high value (5.03 mg%) for sample (a). Carotene values fluctuated widely (.27, .27, .17, .31, .13, .31, .25, .21, .14, .10, .37 mg%). Thiamine values showed little variation, from .42 mg% for sample (k) to .51 mg% for samples (d, e). The nutrient composition of these corn samples will be compared on a dry basis in a future publication.

Rice: The 4 types of samples varied as follows:

A) *Rice, brown*: This sample had a moderately high content of iron (1.83 mg%), thiamine (.16 mg%) and niacin (2.61 mg%).

B) *Rice, polishings* presented very high values of phosphorus (1301.8, 883.0, 893.4 mg%), iron (30.77, 15.29, 12.66 mg%), thiamine (2.12, 1.15, .86 mg%), riboflavin (.32, .18, .18 mg%) and niacin (33.61, 17.20, 14.49 mg%). Sample (a) was usually higher than samples (b) and (c).

C) *Rice, unpolished:* Sample (a) was highest in thiamine (.23 vs. .06, .05 mg%) and in niacin (4.03 vs. 1.38, 1.48 mg%), while sample (c) was highest in iron (16.62 vs. 3.63, 2.86 mg%).

Earth Vegetables

Arracacha: The niacin content of this sample was high, a result in agreement with a previous analysis *(1)*.

Carrot: The carrot is among the richest of plant foods in carotene content. The one sample reported here contained 6.04 mg%.

Radish: This sample contained 39.4 mg% of ascorbic acid, an amount comparable to that of lemons.

Taro: This tuber contains slightly more calcium (100.9 mg%) and iron (1.96 mg%) than other root vegetables.

Fruit Vegetables

Avocado: The ether extract content of the sample of avocado (8.65 g%) was slightly lower than the average content of previously analyzed samples (9.75 g%) (2). This is likely related to degree of ripeness, for it has been observed that the fat content of avocados increases as they ripen.

Peppers: Four samples of pepper were analyzed. The ripe pepper (d) contained 1.89 mg% carotene and .18 mg% riboflavin as compared with the green pepper (c) which contained .25 mg% carotene and .04 mg% riboflavin. On the other hand, the ascorbic acid content of the 4 samples of pepper varied from 75.4 mg% to 281.0 mg%.

Plantain: This food is a significant source of carotene. The 9 samples varied in content from .17 mg% to 1.60 mg%. Ripe plantains of the

"burro" type showed lower nutrient composition than green plantains of the "macho" type.

Squash: The carotene values for the 3 samples analyzed varied from 1.00 mg% for sample (a) to .40 mg% for samples (c).

Tomato, cherry: The cherry tomato contains moderate amounts of carotene (1.05 mg%) and is richer in this nutrient than the samples of ordinary tomatoes.

Vegetable Herbage

Chard: This sample was moderately rich in calcium (104.7 mg%), carotene (1.37 mg%) and ascorbic acid (62.9 mg%).

Chive: This sample was low in most vitamins except carotene (1.37 mg%).

Leek: The leek is a fairly good source of carotene (1.46 mg%), but in other respects is not exceptional.

Lettuce: The 2 samples contained 1.09 mg% and .85 mg% of carotene and 1.47 mg% and 1.98 mg% of iron, in samples (a) and (b), respectively.

Parsley: The sample of parsley contained 138.8 mg% of calcium, 2.28 mg% of iron, 4.42 mg% of carotene, .18 mg% of riboflavin and 146.0 mg% of ascorbic acid. Because it is richer in total protein (N) content, the amino acid content of parsley was nearly twice that of the other herbage vegetables. Parsley is an exceptionally nutritious plant food.

Ramie: The sample analyzed was outstandingly high in calcium (459.0 mg%), carotene (5.99 mg%) and riboflavin (.28 mg%) content and had a moderately high amount of ascorbic acid (73.4 mg%). Though its protein content was higher than that of parsley, it contained much less lysine than parsley.

Ramie has an advantage over parsley in that its flavor is mint, but it is very high in fiber content (2.36 mg%). A sample of less mature ramie would be more acceptable in this respect.

Watercress: The 2 samples were moderately rich in calcium (90.7, 102.0 mg%), iron (1.37, 2.4 mg%), carotene (1.8, 1.6 mg%), riboflavin (.13 mg%) and ascorbic acid (50.4, 50.7 mg%) content. The generally high nutritional value of watercress makes it especially valuable dietetically.

Legumes and Oleaginous Seeds

Beans, black: This sample was rich in calcium (171.5 mg%), iron (9.09 mg%), thiamine (.81 mg%) and riboflavin (.32 mg%). Because of its high protein (N) content, high amounts of tryptophan (243 mg%). methionine (231 mg%) and lysine (1505 mg%) were found.

Peanut: The peanut samples proved to be significant sources of iron, riboflavin, niacin, and amino acids. The roasted samples (b, c) contained .17 mg% and .11 mg% thiamine, whereas the unroasted sample contained 1.54 mg%. It is evident that considerable amounts of thiamine were destroyed by roasting.

Sesame seed: This sample was similar to peanuts in nutrient content. It was rich in calcium (931.0 mg%), iron (10.90 mg%), thiamine (.64 mg%), and fat (45.30 g%) content.

TABLE 1

Composition of food samples from Cuba

Name of food sampled			Constituents measured and yields per 100 g.															
English	Scientific	Spanish	Moisture	Ether Extract	Crude Fiber	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic Acid Total	Tryptophan	Methionine	Lysine
			g.	g.	g.	g.	g.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Cereals																		
Corn	Zea mays L.	Maíz tierno	62.6	1.00	.93	.75	.70	6.6	113.1	1.25	.16	.15	.11	1.70	14.4	16	82	173
Corn, dry (a)	Zea mays L.	Maíz seco	7.4	4.24	2.22	1.88	1.37	10.0	312.0	5.03	.27	.45	.11	1.27	....	48	144	255
Corn, dry (b)	Zea mays L.	Maíz seco	8.8	3.97	2.55	1.65	1.37	9.3	315.0	2.67	.27	.45	.10	1.48	....	48	139	243
Corn, dry (c)	Zea mays L.	Maíz seco	10.1	4.38	2.10	1.79	1.37	10.1	305.0	2.63	.17	.49	.10	1.48	....	48	130	243
Corn, dry (d)	Zea mays L.	Maíz seco	9.0	3.94	1.61	1.75	1.28	8.6	284.5	2.54	.31	.51	.11	1.53	....	48	126	246
Corn, dry (e)	Zea mays L.	Maíz seco	8.8	3.98	2.29	1.78	1.47	10.5	328.0	2.87	.13	.51	.10	1.63	....	53	134	245
Corn, dry (f)	Zea mays L.	Maíz seco	7.5	3.99	1.64	1.69	1.39	11.0	335.5	2.94	.31	.41	.09	1.33	....	49	129	231
Corn, dry (g)	Zea mays L.	Maíz seco	8.9	3.50	2.15	1.90	1.41	9.7	338.5	2.77	.25	.47	.10	1.29	....	45	143	251
Corn, dry (h)	Zea mays L.	Maíz seco	8.8	4.18	2.34	1.79	1.39	7.9	320.2	3.12	.21	.43	.10	1.48	....	45	119	245
Corn, dry (i)	Zea mays L.	Maíz seco	9.7	3.87	2.09	1.66	1.38	7.1	296.1	2.94	.14	.43	.10	1.49	....	49	134	241
Corn, dry (j)	Zea mays L.	Maíz seco	9.5	3.78	2.10	1.65	1.34	7.0	306.4	2.58	.10	.43	.11	1.42	....	47	155	252
Corn, dry (k)	Zea mays L.	Maíz seco	8.4	3.88	2.22	1.72	1.31	7.7	321.3	2.56	.37	.42	.10	1.33	....	52	173	274
Rice, brown	Oryza sativa L.	Arroz integral	13.8	.97	.62	1.22	.75	15.5	184.6	1.83	....	.16	.04	2.61	....	76	145	269
Rice, polishings (a)	Oryza sativa L.	Arroz, polvo de	8.1	1.36	2.87	1.98	.66	105.8	1301.8	30.77	....	2.12	.32	33.61	....	108	205	608
Rice, polishings (b)	Oryza sativa L.	Arroz, polvo de	9.7	7.65	2.78	1.94	.43	61.6	883.0	15.29	....	1.15	.18	17.20	....	111	210	468
Rice, polishings (c)	Oryza sativa L.	Arroz, polvo de	10.7	5.64	2.55	1.92	.41	70.2	893.4	12.66	....	.86	.18	14.49	....	103	225	518
Rice, polished (a)	Oryza sativa L.	Arroz pulido	13.0	.38	.57	1.19	.43	9.0	109.5	.93	....	.09	.03	1.67	....	54	117	210
Rice, polished (b)	Oryza sativa L.	Arroz pulido	12.8	.31	.60	1.21	.33	8.1	102.8	1.16	....	.06	.03	1.40	....	68	117	232
Rice, polished (c)	Oryza sativa L.	Arroz pulido	13.2	.73	.37	1.06	.36	7.2	102.4	1.19	....	.05	.03	1.40	....	44	129	240
Rice, polished (d)	Oryza sativa L.	Arroz pulido	9.4	.60	.62	1.20	.48	6.4	119.3	1.15	....	.09	.03	1.72	....	58	119	229
Rice, polished (e)	Oryza sativa L.	Arroz pulido	9.8	.10	.36	1.00	.41	13.1	69.7	....	....	.04	.03	1.34	....	53	124	209
Rice, polished (f)	Oryza sativa L.	Arroz pulido	9.1	.38	.62	1.17	.45	11.6	126.2	1.19	....	.08	.03	1.63	....	44	108	220
Rice, polished (g)	Oryza sativa L.	Arroz pulido	9.0	.23	.40	1.17	.37	10.8	110.0	1.06	....	.07	.03	1.58	....	51	128	245
Rice, unpolished (a)	Oryza sativa L.	Arroz no pulido	12.9	.95	1.02	1.74	.66	14.9	147.4	3.63	....	.23	.05	4.03	....	97	214	389
Rice, unpolished (b)	Oryza sativa L.	Arroz no pulido	13.0	.23	.67	1.13	.41	15.8	111.3	2.86	....	.06	.03	1.38	....	74	117	200
Rice, unpolished (c)	Oryza sativa L.	Arroz no pulido	13.1	.26	.81	1.07	.62	15.8	61.2	16.62	....	.05	.04	1.48	....	65	126	210
Earth Vegetables																		
Arracacha	Arracacia xanthorrhiza Bancr.	Afió	74.0	.14	.36	.10	1.09	15.0	33.3	.98	.16	.05	.02	3.00	35.8	6	3	26
Beet (a)	Beta vulgaris L.	Remolacha	88.5	.06	.70	.27	.82	18.3	42.2	1.14	.01	.02	.06	.27	1.6	10	2	47
Beet (b)	Beta vulgaris L.	Remolacha	89.4	.04	.62	.24	.78	20.5	23.9	2.81	.01	.01	.04	.18	6.0	11	2	23
Carrot	Daucus carota L.	Zanahoria	91.7	.10	.70	.13	.95	37.9	45.1	1.86	6.04	.05	.04	.73	7.6	9	1	27
Manioc (a)	Manihot esculenta Crantz	Yuca	58.1	.23	.85	.31	.95	49.0	61.0	.90	.02	.08	.04	.66	25.5	34	1	43
Manioc (b)	Manihot esculenta Crantz	Yuca	70.3	.25	.64	.15	.96	63.8	48.1	1.20	.03	.08	.02	.43	30.0	30	1	40
Onion	Allium cepa L.	Cebolla	93.1	.02	.46	.19	.37	38.3	33.9	.58	.01	.05	.03	.21	7.3	16	5	45
Potato (a)	Solanum tuberosum L.	Papa	82.9	.06	.36	.30	.79	13.1	45.6	1.02	.01	.08	.02	1.25	28.1	16	18	91
Potato (b)	Solanum tuberosum L.	Papa	86.8	.08	.29	.28	.67	12.2	44.4	.92	.01	.09	.03	1.26	36.0	15	12	94
Potato (c)	Solanum tuberosum L.	Papa	85.0	.04	.32	.31	.71	9.5	38.6	1.13	.01	.08	.03	1.34	44.9	14	10	100
Radish	Raphanus sativus L.	Rábano	94.7	.02	.48	.12	.69	30.4	32.8	1.44	.01	.02	.05	.37	39.4	6	6	35

TABLE 1—Continued

Composition of food samples from Cuba—Continued

Name of food sampled			Constituents measured and yields per 100 g.														
English	Scientific	Spanish	Moisture	Ether Extract	Crude Fiber	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic Acid Total	Tryptophan	Methionine	Lysine
			g.	g.	g.	g.	g.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Sweet Potato (a)	Ipomoea batatas L.	Boniato	66.5	.25	.80	.27	.77	33.8	36.2	1.31	.02	.12	.04	1.00	27.8	17	16	84
Sweet potato (b)	Ipomoea batatas L.	Boniato	64.2	.15	.69	.37	.62	28.3	29.9	.91	.02	.14	.05	.94	24.1	21	21	111
Sweet potato (c)	Ipomoea batatas L.	Boniato	66.6	.31	.74	.11	.67	44.5	50.7	1.12	.03	.13	.04	.85	34.9	7	8	51
Sweet potato (d)	Ipomoea batatas L.	Boniato	73.7	.13	.80	.11	.75	30.0	48.7	.87	.01	.10	.03	.41	13.4	11	5	26
Taro	Colocasia esculenta Schott	Malanga isleña	75.0	.16	.38	.25	1.21	100.9	71.0	1.96	.01	.08	.03	.90	8.0	24	14	77
Fruit Vegetable																		
Avocado	Persea americana Mill.	Aguacate	81.6	8.65	2.22	.19	.86	8.4	38.5	.79	.17	.04	.12	1.67	23.0	15	11	70
Okra	Hibiscus esculentus (L.) Moench	Quimbombó	89.7	.20	.93	.28	.59	56.6	46.7	.87	.25	.04	.10	.72	23.5	15	17	105
Pepper (a)	Capsicum frutescens L.	Ají cachucha	90.7	.35	.94	.15	.47	18.8	34.4	1.18	.30	.07	.04	.65	75.4	10	5	63
Pepper (b)	Capsicum frutescens L.	Pimiento morrón	91.3	.18	.46	.18	.54	13.8	28.9	.92	.43	.06	.15	.96	167.0	9	7	43
Pepper (c)	Capsicum frutescens L.	Pimiento verde	92.2	.12	.52	.13	.43	12.2	19.6	1.01	.25	.03	.04	.59	248.0	6	2	25
Pepper (d)	Capsicum frutescens L.	Pimiento rojo	89.4	.26	.68	.22	.59	16.6	28.1	1.45	1.89	.04	.18	1.01	281.0	12	7	35
Plantain (a)	Musa paradisiaca L.	Plátano burro	66.7	.13	.51	.36	.77	9.2	37.5	1.09	.43	.06	.05	.41	37.1	11	3	44
Plantain (b)	Musa paradisiaca L.	Plátano burro	66.3	.10	.53	.12	.73	9.1	52.1	.85	.23	.05	.05	.16	42.1	7	6	50
Plantain (c)	Musa paradisiaca L.	Plátano burro	67.2	.05	.45	.14	.72	22.5	32.7	.84	.17	.06	.06	.50	28.5	10	5	48
Plantain (d)	Musa paradisiaca L.	Plátano burro	65.2	.15	.46	.12	.71	8.9	55.1	.93	.27	.06	.05	.52	22.9	8	7	44
Plantain (e)	Musa paradisiaca L.	Plátano macho	59.2	.12	.37	.19	.82	7.6	35.1	.98	1.53	.09	.05	.47	27.7	9	8	56
Plantain (f)	Musa paradisiaca L.	Plátano macho	58.4	.12	.43	.17	.65	7.4	35.2	1.38	1.60	.06	.04	.45	26.0	8	6	52
Plantain (g)	Musa paradisiaca L.	Plátano macho	59.0	.11	.43	.17	.75	10.4	33.8	1.46	1.30	.09	.04	.38	27.2	8	7	55
Plantain (h)	Musa paradisiaca L.	Plátano macho	64.0	.10	.39	.16	.80	10.5	36.7	2.14	.95	.06	.06	.50	28.8	9	10	48
Plantain (i)	Musa paradisiaca L.	Plátano macho	60.4	.17	.40	.19	.70	8.4	34.0	1.16	.91	.08	.05	.53	24.2	10	8	53
Squash (a)	Cucurbita moschata Poir.	Calabaza	81.4	.15	.68	.16	.91	16.4	51.8	.92	1.00	.10	.08	1.19	30.6	16	7	72
Squash (b)	Cucurbita moschata Poir.	Calabaza	91.9	.15	.66	.13	.44	19.0	27.3	.85	.84	.04	.04	.64	14.2	5	0	35
Squash (c)	Cucurbita moschata Poir.	Calabaza	84.2	.23	.62	.13	.58	9.8	43.9	.81	.40	.06	.05	.55	27.7	7	5	38
Tomato, cherry	Lycopersicum esculentum var. cerasiforme Alef.	Tomate placero	92.7	.30	.75	.23	.67	10.2	26.0	.94	1.05	.11	.08	.72	31.0	12	13	63
Herbage Vegetables																		
Cabbage	Brassica oleracea var. capitata L.	Col	92.9	.06	.64	.16	.54	60.3	17.4	.50	.01	.05	.04	.23	42.7	5	2	45
Chard	Beta vulgaris var. cicla L.	Acelga	93.3	.20	.66	.24	.96	104.7	30.3	1.50	1.37	.07	.14	.61	62.9	18	8	76
Chive	Allium schoenoprasum L.	Cebolla de la tierra	92.6	.25	.72	.18	.61	75.9	26.2	.98	1.37	.04	.11	.31	22.5	14	9	93
Leek	Allium porrum L.	Ajo puerro	91.1	.36	.95	.23	.85	62.4	30.6	1.62	1.46	.08	.10	.47	32.1	15	10	91
Lettuce (a)	Lactuca sativa L.	Lechuga	94.9	.24	.37	.21	.71	34.6	24.7	1.47	1.09	.07	.09	.45	30.9	15	7	100
Lettuce (b)	Lactuca sativa L.	Lechuga	95.9	.25	.32	.15	.52	49.6	24.9	1.98	.85	.06	.10	.41	21.2	16	8	73
Parsley	Petroselinum crispum Mansf.	Perejil	89.0	.48	1.14	.47	1.59	138.8	62.7	2.28	4.42	.09	.18	.98	146.0	34	14	237
Ramie	Boehmeria nivea L.	Ramié	87.0	.46	2.36	.53	2.08	459.0	45.4	1.65	5.99	.03	.28	.73	73.4	43	24	157
Watercress (a)	Nasturtium officinale R. Br.	Berro	95.2	.23	.56	.27	.74	90.7	29.5	1.30	1.82	.06	.13	.61	50.4	28	7	102
Watercress (b)	Nasturtium officinale R. Br.	Berro	94.7	.20	.69	.32	.77	102.0	29.4	2.40	1.64	.06	.13	.77	53.7	25	6	90

TABLE 1—Continued

Composition of food samples from Cuba—Continued

Name of food sampled			Constituents measured and yields per 100 g.														
English	Scientific	Spanish	Moisture	Ether Extract	Crude Fiber	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic Acid Total	Tryptophan	Methionine	Lysine
			g.	g.	g.	g.	g.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Legumes and Oleaginous Seeds																		
Bean, black	Phaseolus vulgaris L.	Frijol negro	13.4	.87	3.95	3.58	3.52	171.5	420.0	9.09	.01	.81	.32	2.20	....	243	231	1505
Bean, snap green	Phaseolus vulgaris L.	Habichuela catalana	92.3	.08	1.00	.30	.54	33.9	38.0	1.05	.42	.06	.11	.54	21.6	16	7	95
Peanut (a)	Arachis hypogaea L.	Maní	15.2	41.60	5.16	3.70	2.06	67.3	399.4	5.64	.00	1.54	.31	13.60	2.8	307	244	1140
Peanut (b)	Arachis hypogaea L.	Maní tostado	1.8	47.60	3.40	5.10	2.49	55.1	480.5	4.07	....	.17	.40	18.00	....	341	347	940
Peanut (c)	Arachis hypogaea L.	Maní tostado	2.1	46.60	3.75	5.20	2.57	59.5	494.5	6.82	....	.11	.38	15.30	....	332	311	1080
Sesame seed	Sesamum indicum L.	Ajonjolí	6.2	45.30	8.44	3.24	5.37	931.0	716.5	10.90	....	.64	.28	7.15	....	284	553	689
Fruit: Citrus																		
Grapefruit (a)	Citrus paradisi Macfad.	Toronja	89.9	.08	.18	.13	.34	9.6	22.5	.55	.01	.07	.03	.23	49.3	2	1	17
Grapefruit (b)	Citrus paradisi Macfad.	Toronja	89.6	.08	.19	.10	.29	14.7	17.7	.42	.12	.05	.02	.19	36.6	3	2	15
Grapefruit (c)	Citrus paradisi Macfad.	Toronja	90.6	.08	.18	.07	.26	8.6	11.8	.82	.01	.05	.02	.19	49.7	2	2	17
Lime (a)	Citrus aurantifolia Swingle	Lima	91.1	.06	.20	.11	.30	15.3	15.1	.54	.00	.05	.03	.16	66.8	4	2	21
Lime (b)	Citrus aurantifolia Swingle	Lima	91.5	.07	.19	.10	.25	8.5	16.5	.48	.00	.04	.02	.13	49.0	3	1	14
Lime (c)	Citrus aurantifolia Swingle	Limón	90.3	2.80	.09	.06	.23	11.1	13.1	.37	.01	.01	.03	.12	41.8	2	1	9
Lime (d)	Citrus aurantifolia Swingle	Limón	89.3	2.82	.06	.06	.15	8.7	9.1	.53	.01	.01	.02	.06	42.4	2	1	8
Lime (e)	Citrus aurantifolia Swingle	Limón	90.9	2.70	.07	.06	.15	8.2	10.4	.44	.01	.02	.02	.06	29.4	1	0	6
Lime (f)	Citrus aurantifolia Swingle	Limón	90.7	2.62	.05	.05	.13	5.1	9.1	.27	.01	.01	.01	.08	32.5	1	0	4
Lime (g)	Citrus aurantifolia Swingle	Limón	90.2	2.57	.07	.08	.20	7.6	16.2	.47	.02	.01	.01	.08	36.3	2	0	5
Lime (h)	Citrus aurantifolia Swingle	Limón	90.5	.36	.08	.05	.14	6.2	10.5	1.38	.01	.01	.02	.05	31.0	1	0	6
Orange (a)	Citrus sinensis (L.) Osbeck.	Naranja	86.9	.04	.19	.11	.36	13.5	23.6	.93	.06	.07	.05	.24	40.9	5	1	22
Orange (b)	Citrus sinensis (L.) Osbeck.	Naranja	91.5	.05	.04	.14	.35	8.7	21.4	.69	.01	.08	.03	.20	41.1	2	2	17
Orange, mandarin (a)	Citrus reticulata Blanco.	Mandarina	88.6	.29	.39	.11	.38	34.7	16.0	.49	.20	.11	.03	.28	29.0	7	5	27
Orange, mandarin (b)	Citrus reticulata Blanco.	Mandarina	86.5	.25	.34	.07	.28	18.6	10.9	.56	.01	.05	.03	.19	45.0	3	2	20
Orange, sour (a)	Citrus aurantium L.	Naranja agria	89.2	.11	.14	.07	.45	12.8	11.4	.41	.01	.03	.03	.12	39.1	1	1	11
Orange, sour (b)	Citrus aurantium L.	Naranja cajel	90.6	.05	.14	.08	.40	22.3	19.8	.82	.01	.09	.04	.27	62.8	3	1	17
Fruit: Other																		
Antidesma	Antidesma platyphyllum H. Mann.	Antidesma	80.7	.09	.13	.07	.85	16.0	41.9	1.82	.15	.01	.03	.54	17.0	4	2	14
Banana, apple	Musa paradisiaca var. sapientum Ktze.	Plátano manzano	68.6	.16	.33	.17	.60	8.6	34.4	.80	.08	.05	.05	.63	21.7	13	4	53
Barbados gooseberry	Pereskia aculeata Mill.	Grosellero de la Florida	96.8	.11	.16	.11	.35	13.1	8.8	.82	2.04	.02	.03	.99	4.3	8	0	12
Carambola	Averrhoa carambola L.	Carámbola	89.9	.08	.80	.06	.26	4.9	21.0	.50	.07	.03	.03	.38	53.1	3	2	26
Cashew apple	Anacardium occidentale L.	Marañón	90.4	.02	.04	.05	.19	4.2	6.1	.69	.00	.00	.02	.13	372.0	1	0	6
Coconut meat	Cocos nucifera L.	Coco, masa de	57.1	23.90	3.84	.57	1.00	19.6	93.8	2.17	....	.02	.02	.66	3.7	23	100	261
Coconut milk	Cocos nucifera L.	Coco, agua de	91.4	.21	.00	.02	.45	23.6	14.5	.70	....	.00	.01	.28	2.4	0	0	2
Custard apple	Annona reticulata L.	Mamón	77.0	.02	2.67	.30	.66	25.5	26.0	.96	.01	.09	.10	.42	40.5	8	4	41

TABLE 1—Concluded

Composition of food samples from Cuba—Concluded

Name of food sampled			Constituents measured and yields per 100 g.														
English	Scientific	Spanish	Moisture	Ether Extract	Crude Fiber	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic Acid Total	Tryptophan	Methionine	Lysine
			g.	g.	g.	g.	g.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Garcinia, sweet	Garcinia dulcis Kurz.	Garcinia	86.7	.50	1.04	.06	.25	4.6	13.3	.45	.06	.06	.04	.26	5.3	4	4	24
Guava (a)	Psidium guajava L.	Guayaba	80.5	.85	6.22	.14	.54	17.7	25.0	.93	.05	.06	.05	.82	152.0	9	7	33
Guava (b)	Psidium guajava L.	Guayaba	80.8	.96	5.76	.14	.51	25.9	26.9	.92	.28	.05	.05	.88	255.0	8	7	30
Guava (c)	Psidium guajava L.	Guayaba	81.1	1.00	5.26	.14	.51	20.7	30.4	.90	.13	.05	.04	.65	283.0	7	7	26
Guava (d)	Psidium guajava L.	Guayaba	80.3	1.15	5.35	.14	.59	24.5	35.4	1.01	.12	.05	.05	.65	417.0	8	8	28
Guava (e)	Psidium guajava L.	Guayaba	81.5	.77	5.16	.13	.44	15.8	21.5	.99	.25	.04	.05	.71	189.0	9	3	22
Guava (f)	Psidium guajava L.	Guayaba	82.2	.78	4.46	.17	.45	13.7	26.5	.85	.02	.05	.05	.26	33.5	10	5	38
Honeyberry	Melicocca bijuga L.	Mamoncillo	81.4	.08	.07	.10	.34	6.7	9.8	1.19	.00	.13	.02	.30	1.5	14	0	17
Jaboticaba	Myrciaria cauliflora (DC) Berg.	Jaboticaba	87.1	.01	.08	.01	.20	6.3	9.2	.49	.00	.02	.02	.21	22.7	1	0	7
Mamey	Mammea americana L.	Mamey de Santo Domingo	88.8	.15	.81	.08	.27	10.7	15.5	.52	.37	.03	.06	.31	11.8	5	5	34
Mango (a)	Mangifera indica L.	Manga amarilla	84.4	.13	.57	.08	.37	6.8	10.2	.75	1.32	.08	.06	.48	48.1	6	5	38
Mango (b)	Mangifera indica L.	Manga blanca	86.6	.03	.40	.04	.29	8.0	13.5	.48	.24	.03	.04	.45	5.4	2	1	14
Mango (c)	Mangifera indica L.	Mango mamey	84.8	.05	.84	.06	.31	7.7	9.1	.72	1.07	.03	.04	.35	57.9	4	3	29
Mango (d)	Mangifera indica L.	Mango Toledo	79.2	.08	.68	.08	.32	6.5	12.1	.39	.80	.04	.05	1.04	80.5	5	5	31
Melon	Cucumis melo L.	Melón de Castilla	94.3	.08	.25	.08	.30	8.2	9.0	.59	.46	.02	.02	.63	27.5	3	0	14
Myrobalan	Phyllanthus emblica L.	Mirobalanos	77.1	.20	1.86	.07	.51	12.5	26.0	.48	.01	.03	.05	.18	625.0	3	2	17
Paniala	Flacourtia cataphracta Roxb.	Ciruela Gobernadora	69.5	.04	.52	.08	.54	20.1	43.7	1.19	.03	.02	.03	.56	13.6	7	4	34
Papaya (a)	Carica papaya L.	Fruta bomba	93.2	.05	.73	.05	.30	19.0	6.8	.73	.18	.02	.03	.18	31.3	3	0	12
Papaya (b)	Carica papaya L.	Fruta bomba	92.4	.05	.84	.08	.39	12.3	19.1	.55	.20	.02	.02	.28	46.1	5	0	19
Papaya (c)	Carica papaya L.	Fruta bomba	92.0	.05	.81	.05	.23	13.9	9.7	.59	.13	.02	.02	.21	23.1	3	1	13
Pineapple (a)	Ananas comosus Merr.	Piña	83.8	.05	.43	.06	.21	24.9	13.5	.47	.02	.11	.05	.59	37.2	6	3	16
Pineapple (b)	Ananas comosus Merr.	Piña	84.1	.05	.46	.09	.31	14.5	10.4	.55	.02	.12	.06	.23	18.0	6	6	24
Sapodilla	Achras zapota L.	Níspero	78.4	.51	1.52	.06	.38	11.8	16.4	.84	.03	.00	.05	.17	21.0	3	2	29
Sapote (a)	Calocarpum sapota Merr.	Mamey colorado	65.4	.12	1.34	.25	1.06	30.0	31.6	1.04	1.14	.01	.08	2.41	29.7	23	14	90
Sapote (b)	Calocarpum sapota Merr.	Mamey colorado	69.0	.07	1.08	.38	.92	39.6	20.2	1.11	.68	.02	.11	2.27	35.3	16	11	90
Star-apple (a)	Chrysophyllum cainito L.	Caimito	80.1	1.27	.58	.11	.42	18.3	17.2	.74	.01	.08	.05	.61	14.1	4	2	18
Star-apple (b)	Chrysophyllum cainito L.	Caimito	85.5	1.60	.53	.12	.37	11.0	16.6	.63	.01	.08	.04	.60	10.7	5	2	26
Sweetsop	Annona squamosa L.	Anón	74.0	.12	1.63	.22	.68	29.5	34.0	1.23	.00	.11	.09	.70	50.6	10	6	53
Tamarind	Tamarindus indica L.	Tamarindo	31.1	.11	1.27	.50	1.91	30.9	119.6	2.30	.01	.37	.19	2.14	2.4	20	16	155
Watermelon	Citrullus vulgaris Schrad.	Melón de agua	94.0	.05	.23	.09	.18	4.8	9.1	.43	.11	.02	.03	.20	4.9	5	1	12
Miscellaneous																		
Guava, marmalade	Psidium guajava L.	Guayaba, mermelada de	50.0	.06	1.43	.08	.35	13.4	13.5	.97	.03	.01	.04	.52	53.7	4	1	11
Guava, pulp	Psidium guajava L.	Guayaba, pulpa de	86.8	.08	2.42	.10	.49	19.5	19.5	1.80	.23	.02	.05	.89	131.0	6	3	22
Guava, shells (a)	Psidium guajava L.	Guayaba, cascos de	80.8	.08	2.92	.06	.21	20.9	7.3	1.95	.08	.00	.02	.17	13.1	4	1	16
Guava, shells (b)	Psidium guajava L.	Guayaba, cascos de	52.6	.07	2.75	.05	.21	21.7	9.1	.74	.01	.01	.02	.22	38.8	3	0	9
Molasses, blackstrap	Saccharum hybrid	Miel final	16.2	.13	.31	.37	5.20	652.5	44.0	33.50	....	.04	....	1.09	....	7	4	19

Fruit, Citrus

Grapefruit: Of the 3 grapefruits analyzed, samples (a) and (c) were higher in ascorbic acid (49.3 and 49.7 mg%) than sample (b) (36.6 mg%).

Lime: Of the different classes of lime analyzed, the so-called lime (a, b) contained 66.8 mg% and 49.0 mg% ascorbic acid, whereas the other varieties (d, e) contained between 42.4 mg% to 29.4 mg%.

Orange: The 2 samples analyzed showed almost the same amount of ascorbic acid (40.9, 41.1 mg%).

Orange, sour: The 2 samples analyzed were quite different in ascorbic acid content (39.1, 62.8 mg%). It is interesting that the sour orange is not greatly different in ascorbic acid content as compared with the regular orange.

Other Fruits

Barbados gooseberry: This fruit contained an important amount of carotene (2.04 mg%) but otherwise was not outstanding.

Cashew apple: The sample was extremely high in ascorbic acid content (372.0 mg%). Thus it ranks among the best food sources of this vitamin.

Coconut: The sample of coconut meat was rich in lipids (23.90 g%), but this value was less than was found in earlier samples *(1)*. The coconut milk was richer in calcium than the meat (23.6 mg% vs. 19.6 mg%) but the iron content of the meat was much higher (2.17 vs. .70 mg%). Coconut meat is nutritious.

Guava: The 6 samples analyzed showed great variation in ascorbic acid, from 33.5 mg% to 417.0 mg%. The lowest value corresponded to sample (f) the so-called "guayaba del Peru." The fiber content was very high in all samples, 4.46 to 6.22 g%.

Mango: The carotene content of the 4 samples analyzed varied between .24 mg% and 1.32 mg%. Sample (b) was a white mango, a name confirmed by its low carotenoid content. The ascorbic acid value also showed wide variations, 5.4 to 80.5 mg%, with the lowest value again in sample (b). The niacin value for sample (d) (1.04 mg%) was higher than that of the other three samples (.48, .45, .35 mg%).

Myrobalan: This sample had the highest ascorbic acid value found to date in Cuban food (625 mg%). In other respects this fruit does not appear to be significant.

Papaya: The 3 samples contained 23.1 mg% to 46.1 mg% ascorbic acid.

Pineapple: The 2 samples were low in ascorbic acid content (37.2, 18.0 mg%) in comparison with values obtained previously *(1)*.

Sapote: The 2 samples were richer in carotene (1.14 and .68 mg%) and niacin (2.41, 2.27 mg%) than many other fruits. Their ascorbic acid content was similar to that of lemons (29.7, 35.3 mg%).

Tamarind: This fruit is not especially nutritious. It contains fair amounts of niacin (2.14 mg%) and iron (2.30 mg%), but is otherwise unimportant.

Miscellaneous

Guava, processed: Guava pulp contained only one-third as much ascorbic acid (131.0 mg%) as the average content of the fresh fruit *(2)*.

Molasses, blackstrap: Blackstrap molasses is outstanding as a source of calcium (652.5 mg%) and iron (33.50 mg%).

SUMMARY

A total of 137 samples of Cuban foods of vegetable origin were analyzed for content of the following 16 nutrients: moisture, ether extract, crude fiber, nitrogen, ash, calcium, phosphorus, iron, carotene, thiamine, riboflavin, niacin, ascorbic acid, tryptophan, methionine, and lysine.

The samples were collected during the period between February and December 1954. Four were obtained in markets, and the remainder from farms and gardens throughout the island.

The analytical data are presented and evaluated.

Peanuts are rich in protein, fat, thiamine, riboflavin, niacin, tryptophan, and lysine. Sesame seed is rich in protein, fat, fiber, calcium, iron, riboflavin, tryptophan, methionine, and lysine. Myrobalan, pepper, guava, and the cashew apple are extremely high in content of ascorbic acid. Molasses is very rich in calcium and iron. Rice polishings are rich in protein, fat, iron, thiamine, riboflavin, niacin, tryptophan, methionine, and lysine. Parsley is rich in calcium, carotene, and ascorbic acid. Ramie is rich in calcium, carotene, and riboflavin. Coconut meat is rich in fat, methionine, and lysine.

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NUTRITIVE VALUE OF FISH [a]. I. NICOTINIC ACID, RIBOFLAVIN, VITAMIN B_{12}, AND AMINO ACIDS OF VARIOUS SALT-WATER SPECIES

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The consumption of fish and other marine products has always been an important factor in the economy and in the nutrition of coastal populations. Recently there has been a trend toward increased fish consumption, particularly in school lunch programs (*21*, *25*, *27*). Fish cookery demonstrations before school lunch personnel in Maine, New Hampshire, Vermont, Rhode Island, and Connecticut resulted in a 59% increase in fish consumption in the school lunch program. It is predicted that one result of this campaign will be an increased use of fish in the area as a whole (*25*), which, considering the high nutritive value of fishery products, should benefit the nutritional status of the population.

Available information on the vitamin and amino acid content of fish includes reports on the nutritive value of fish from Michigan waters (*19*, *22*, *23*), the levels of certain vitamins in Florida-produced foods (*12*), and the B-vitamin content of certain marine products (*1*, *17*). Studies of specific nutrients in certain fishery products have include ascorbic acid (*11*, *14*), thiamine (*13*, *23*, *35*), nicotinic acid (*6*, *22*, *26*), riboflavin (*2*, *15*, *18*), vitamin B_{12} (*10*, *16*, *30*, *34*, *38*), and various amino acids (*7*, *9*, *28*, *29*, *33*). Similar information with respect to the nicotinic acid, riboflavin, vitamin B_{12}, and amino acids of various species commonly consumed in New England and other areas is reported herein. Included are certain marine products, such as lobsters, which are of particular importance to the New England area.

EXPERIMENTAL PROCEDURE

Market-fresh samples of various species of fish, including shellfish, which are commonly consumed in New England, were analyzed qualitatively for amino acids and quantitatively for nicotinic acid, riboflavin, vitamin B_{12}, total nitrogen, and total solids. A small number of canned samples was also analyzed.

The edible portion of the fish muscle, blended in a Waring blender, was used for analysis. With the small fish, several were blended together. The vitamin analyses were carried out on hot-water extracts (autoclaved) of the blended mixtures by standard quantitative microbiological procedures (*8*), using *Lactobacillus arabinosus* 17-5 (A. T. C. C. No. 8014) for nicotinic acid, *Lactobacillus casei* (No. 7469) for riboflavin, and *Lactobacillus leichmannii* (No. 7830) for vitamin B_{12}. Protein was determined by the macro-Kjeldahl nitrogen procedure on 2-g. aliquots of the blended fish, and moisture determinations were made by heating weighed samples in an oven at approximately 103° C. for 48 hours.

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The distribution of amino acids was determined by paper chromatography (*4*). A 5-g. aliquot of the blended fish sample was hydrolyzed under a reflux condenser in a final concentration of approximately 6 *N* HCl at 100° C. for 24 hours. The hydrolyzed sample was filtered and deacidified with amberlite IR-4B (OH) resin by the batch method (*24*). The deacidified solution was decanted from the resin and washed to a total volume of 500 ml. Two hundred ml. of the diluted solution was evaporated to a final volume of 10 ml. This concentrated solution was used for chromatography.

Tryptophan may not be detected after acid hydrolysis. Therefore, it was necessary to subject another portion of the blended fish to alkaline hydrolysis. A 5-g. aliquot of the blended fish was hydrolyzed with 40 ml. of 5 *N* NaOH. Hydrolysis was carried out as above. After hydrolysis, the solution was filtered. An aliquot was neutralized with HCl and centrifuged. An aliquot of the filtrate was used for chromatography.

Chromatographic procedure. Aliquots of the fish hydrolysates were subjected to two directional ascending chromatography (37), using phenol-water and collidine-lutidine-water as the resolving solutions, followed by ninhydrin spray to detect the amino acids. This method detected most of the amino acids, including those occurring in small amounts.

The Pauli test (*3*) for histidine and tyrosine and the Sakaguchi test (*20*) for arginine were applied to chromatograms run in butanol-acetic acid-water (v/v) (4-1-1) in the descending method.

Chromatography of an aliquot of the alkaline hydrolysate was done in one direction, using the above butanol-acetic acid-water resolving solution. Tryptophan was detected by the Ehrlich test (*3*).

In order to determine the relative amounts of the amino acids in the fish proteins, the size and intensity of the spots on the chromatograms were compared with those of standard concentrations of known amino acids. A two-directional chromatogram without further treatment does not allow for quantitative estimation of all of the spots, since there is some overlapping of spots. The following procedures were also used in the comparisons.

A 2-ml. aliquot of the acid hydrolysate was passed through a cation exchange column of 1RC-50 prepared at pH 7 (*2*). The basic amino acids, arginine and lysine, were adsorbed on the column and the neutral and acidic amino acids passed through. The column was washed with water and the eluate made to 100 ml. Upon evaporation of the eluate, two different aliquots of the concentrated solutions were chromatographed in two directions simultaneously with standard aliquots of aspartic and glutamic acids, serine, gycine, alanine, and threonine.

Several different aliquots of the concentrated acid hydrolysates were chromatographed, using 3 successive descending resolutions in butanol-acetic acid-water (4-1-1) simultaneously with standard amounts of leucine, valine, methionine, phenylalanine, proline, and cystine. The amino acids were detected with isatin (*31*).

RESULTS AND DISCUSSION

In Table 1 are presented, in the form of averages, the results of the moisture, protein, nicotinic acid, riboflavin, and vitamin B_{12} determinations on the various species. Although it was not true in all cases, there were some species which gave rather wide variations in results. These included nicotinic acid in swordfish (50-110 mcg./g.), halibut (36.9-102), salmon (57.5-100), and mackerel (55-92.4); and vitamin B_{12} in salmon (18-50 millimcg./g.), smelts (18-48), mackerel (30-67), clams (300-1,250), oysters (83-197). It is felt that some of this variation might have been related to a seasonal factor, as samples were obtained at different seasons of the year. Such a result has been reported for fresh-water fish (*22*).

The results indicate, in general, the high nutritive value of these marine species. Total protein is particularly high in halibut, mackerel, salmon, swordfish, lobsters, shrimp, canned salmon, and tuna. The lower total

protein results exhibited by the other species are, for the most part, accounted for by higher moisture contents.

As a source of nicotinic acid, the following species can be considered as equal to or better than beef: halibut, mackerel, salmon, swordfish, and tuna. Haddock and shrimp are also good sources of this vitamin. Haddock roe, mackerel, smelts, clams, oysters, salmon, and tuna apparently are equal to beef as a source of riboflavin. The species showing the highest vitamin B_{12} contents include haddock roe, mackerel, salmon, smelts and, particularly, clams and oysters.

The chromatograms of the hydrolyzed fish proteins showed a similar pattern of amino acid distribution for all the species analyzed. The essential amino acids were found present in all species. A probable absence of cystine was noted in clams and oysters.

The following listing shows the relative distribution of some of the amino acids in the hydrolyzed fish proteins. There has been no distinction of spots for leucine and isoleucine. Amino acids in highest amounts:

leucine(s)	alanine
aspartic acid	lysine
glutamic acid	

Amino acids in moderate amounts:

serine	threonine	arginine
glycine	valine	phenylalanine

Amino acids in low amounts:

methionine	cystine
proline	tryptophan

Tyrosine and histidine, while not quantitatively estimated, were also found present in all species. The amount of tyrosine in clams, however, was particularly low. A chromatogram of mackerel showed an unusually high histidine content, which is in accord with the finding of Shewan (*32*), that the pelagic species contain a preponderance of basic amino acids, especially histidine.

β-alanine was found present in cod, cusk, haddock, swordfish, halibut, salmon, and tuna. It was usually absent in the shellfish, except oysters, although later analyses of clams revealed the occasional presence of β-alanine in this species also.

In the two-directional chromatograms, there were a few unidentified spots occurring spasmodically and in low concentrations. One of these unknowns, however, was present in considerable quantity. It was identified as taurine after the following observations: The location of the spot coincided with that of known taurine. It withstood the copper treatment (*5*) and was, therefore, not an alpha-amino acid. Aliquots of the acid hydrolysates of some of the species which contained this substance were diluted and put through two successive columns of 1:2 cupric carbonate-alumina (w/w) (*36*). The eluates were subjected to one- and two-directional chromatography. In both cases, spots coincided with known taurine which was subjected to the same test.

The taurine spot, present in the shellfish and to some extent in other fish, was most prominent in the molluscs. Its presence was particularly noted in the species having little or no β-alanine.

SUMMARY

Various species of salt-water fish, including shellfish, commonly consumed in the New England area, were analyzed for moisture, protein, nicotinic acid, riboflavin, and vitamin B_{12}. A study of the amino acid distribution was also made. It is concluded that, in general, these salt-water species are similar and in some instances superior to lean meat in nutritive value with respect to the factors studied. The species of particularly high nutritive value include tuna, salmon, mackerel, swordfish, and halibut. Clams and to a lesser extent oysters are an exceptionally good source of vitamin B_{12}.

TABLE 1

Average protein and B vitamin content of various species (fresh weight basis) [1]

Species	Moisture	Protein[2]	Nicotinic Acid	Riboflavin	Vitamin B_{12}
	%	%	mcg./g.	mcg./g.	milli-mcg./g.
Cod	81.8	16.8	19.8	0.31	4.5
Cusk	80.7	17.4	22.0	0.32	3.0
Haddock	81.2	17.7	30.2	0.53	5.3
Haddock Roe	70.5	24.6	13.5	6.90	51.0
Halibut	79.7	18.6	62.1	0.51	7.1
Mackerel	71.4	20.1	76.2	2.51	48.5
Salmon	75.0	20.0	71.9	0.99	28.9
Smelts	80.2	15.6	14.5	1.20	34.4
Swordfish	76.7	19.3	73.9	0.50	5.7
Molluscs:					
Clams	87.9	8.5	12.6	1.88	623.0
Oysters	87.5	6.8	13.1	1.66	146.0
Scallops	79.7	16.4	11.5	0.65	13.4
Crustaceans:					
Lobster (claw)	78.5	17.3	12.4	0.46	4.6
Lobster (tail)	76.4	20.3	16.7	0.50	4.9
Shrimp	78.4	19.9	31.2	0.27	9.1
Canned fish:					
Crab	80.1	16.3	13.7	0.84	4.6
Salmon	66.2	22.4	56.3	1.98	20.7
Tuna	69.6	28.3	141.0	1.05	14.8

[1] Each figure represents 4 to 11 samples.
[2] Protein=N x 6.25.

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PROTEOLYTIC ENZYMES FROM *PSEUDOMONAS PUTREFACIENS*. I. CHARACTERISTICS OF AN EXTRACELLULAR PROTEOLYTIC ENZYME SYSTEM[a]

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Recently, numerous studies have been conducted on the bacterial flora of milk and milk products stored under refrigeration for extended periods. In many instances large numbers of psychrophilic bacteria were found in these products after several days of storage at refrigerator temperatures. Certain psychrophiles when present in considerable numbers break down milk protein or fat and cause undesirable flavors and odors. Others, which do not produce these defects, may raise the total viable population above the legal maximum.

Pseudomonas putrefaciens is one of the psychrophilic organisms frequently isolated from dairy products. Previous investigations *(5, 6)* have shown that many strains of this species are capable of causing protein breakdown in milk. However, little information has been available as to the nature of the proteolytic enzyme system of *Ps. putrefaciens*. In the present investigation, some of the characteristics of the extracellular proteinases of *Ps. putrefaciens* were determined.

EXPERIMENTAL METHODS

Ps. putrefaciens, strain 12, was selected from a group of pure cultures on the basis of its strong proteolytic action in milk after incubation for 3 to 4 days at 25° C. The cultures were identified by reference to Breed *et al. (3)*. The stock cultures were carried in sterile litmus milk at 25° C. and were transferred weekly. Cultures were transferred daily for two transfers prior to each experiment. One per cent inoculum was used with incubation at 25° C. The milk used in the following experiments consisted of fresh skimmilk which was autoclaved at 15 lb. steam pressure for 15 min.

Extracellular enzyme preparation. Sterile skimmilk, inoculated with *Ps. putrefaciens*, was incubated at 25° C. and pH 7.0 for 3 to 4 days. At this time a clear yellowish brown supernatant was formed which was recovered by centrifugation at 5,000 r.p.m. for 30 min. in a refrigerated centrifuge. The clean supernatant then was filtered through a Seitz filter and stored in a freezer and used within a few days.

Determination of proteolytic activity. Sterile, buffered 2% solutions of casein, lactalbumin, α- and β-casein were used as substrates. They were prepared as described in a previous publication (7). The enzyme-substrate mixture consisted of 5 ml. of substrate, enzyme preparation, and sterile distilled water to make a total volume of 10 ml. Unless stated otherwise, 1 ml. of enzyme preparation was incubated with the substrate at pH 7.0 and 37.5° C. for 3 hours. After incubation, nitrogen fractions insoluble in trichloroacetic acid were precipitated as described in a previous paper (7). Nitrogen fractions soluble and insoluble in trichloroacetic acid and total nitrogen were determined by a micro-Kjeldahl procedure (*4*). Unless stated otherwise, proteolysis was expressed as the decrease in trichloroacetic acid (TCA) insoluble protein per ml. of

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the enzyme assay mixture, following incubation for 3 hours at pH 7.0 and 37.5° C. Bacterial counts were made according to *Standard Methods for the Examination of Dairy Products* (*1*). The plates were incubated for 3 days at 25° C.

RESULTS AND DISCUSSION

The effect of the length of the incubation period on the production of extracellular proteinases in a milk culture of *Ps. putrefaciens* was established by determining the proteolytic activity of the cell-free growth medium after incubation periods ranging from 3 to 10 days. As shown in Figure 1, maximum activity against casein and lactalbumin was observed

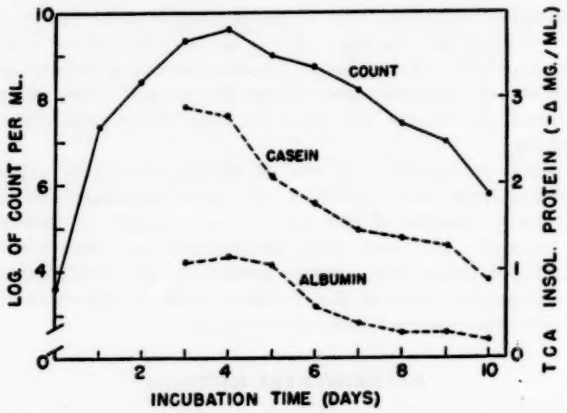

Fig. 1. Effect of age of culture on the proteolytic activity of the cell-free growth medium against casein and albumin (1 ml. of enzyme preparation incubated with substrate at pH 7.0 and 37.5°C. for 3 hours).

after incubation for 3 to 4 days, when the viable cell population had reached a maximum. A gradual decrease in activity took place throughout the rest of the incubation period. The activity against lactalbumin was much less than against casein.

The activity which might have been present during the first 2 days was not determined, because of the difficulty of obtaining a satisfactory cell-free growth medium. The gradual decrease in activity after incubation for 3 to 4 days was not due to a change of pH of the growth medium, since the pH was kept constant throughout the experiment Subsequent experiments on the stability of the enzyme preparation at 25° C. (Table 1) which show a loss of activity after storage at pH 7.0 for 24 to 48 hours might offer an explanation for this phenomenon. In this experiment, portions of the cell-free growth medium obtained after incubation for 3 days at 25° C. were adjusted to pH values ranging from 5.0 to 9.0 and stored at 0° and 25° C. for 24 and 48 hours. Only very little decrease in activity was observed against casein when the cell-free growth medium was stored between pH 5.0 and 9.0 at 0° C. However, at 25° C. a considerable loss of activity was observed even at pH 7.0.

TABLE 1

Stability of enzyme preparation after storage at 0° and 25° C. at various pH levels

pH of enzyme preparation	TCA insol. protein (—Δmg./ml.)[1] after storage at :			
	0° C.		25° C.	
	24 hr.	48 hr.	24 hr.	48 hr.
5.0	2.3	2.2	1.2	0.8
6.0	2.5	2.0	1.2	0.6
7.0	2.7	2.7	1.4	0.6
8.0	2.7	2.6	1.4	0.7
9.0	2.4	2.4	1.2	0.7

[1] On casein substrate. Control at pH 7.0:—2.8 mg./ml.

In Figure 2 are presented data showing the effect of pH on the digestion of casein and lactalbumin. Maximum activity against casein was observed at pH 7.0. Appreciable activity, however, was noted over a pH range of 6.5 to 8.0. The optimum pH for the activity against lactalbumin was approximately 7.5 to 8.0. As shown in Figure 3, the rate of proteolysis rose rapidly with increasing concentration of enzyme preparation up to 1 ml. With greater amounts of enzyme preparation, the rate increased only slowly. The possibility exists that accumulation of end-products is responsible for this phenomenon. Figure 4 shows the effect of incubation temperature (substrate-enzyme mixture) on the digestion of casein and lactalbumin. Maximum activity against casein was observed at 37.5° C; for lactalbumin at 45° C. A sharp decrease in activity took place at temperatures above 45° C. Little or no activity was noted at either 5 or 55° C.

As shown in Figure 5, the rate of proteolytic activity against both casein and lactalbumin increased greatly up to a concentration of 20 mg. per ml. With greater concentrations of substrate, however, the rate increased only slowly. Figure 6 shows the effect of time of incubation (en-

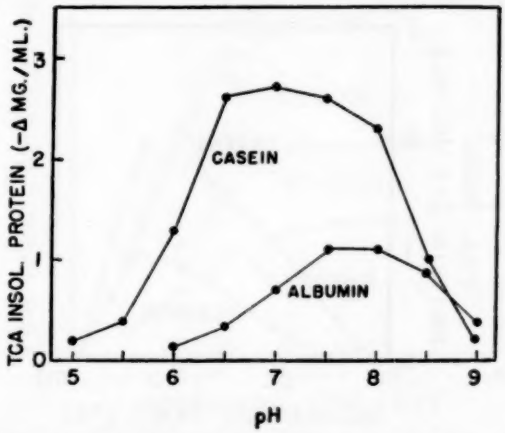

Fig. 2. Effect of pH of substrate on the proteolysis of casein and albumin (1 ml. of enzyme preparation incubated with substrate at 37.5°C. for 3 hours).

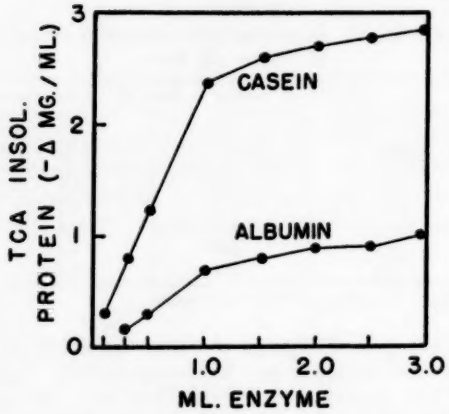

Fig. 3. Effect of quantity of enzyme preparation on the proteolysis of casein and albumin (enzyme preparation incubated with substrate at pH 7.0 and 37.5° C. for 3 hours).

zyme-substrate mixture) on the digestion of casein, α- and β-casein, and lactalbumin. Up to 3 hours of incubation, a direct relationship was apparent between incubation time and digestion of the casein and lactalbumin substrates. The rate of digestion of α-casein was very similar to that of the commercial casein substrate. After 4 hours of incubation, further digestion of the substrates took place at a very slow rate.

Effect of reducing agents and metallic ions. The literature reveals several studies reporting that certain proteolytic enzymes need reducing agents or metallic ions for maximum activity *(2, 7, 8, 9)*. One-ml. quantities of sodium thioglycolate, Na_2SO_3, KCN, ascorbic acid, and cysteine in

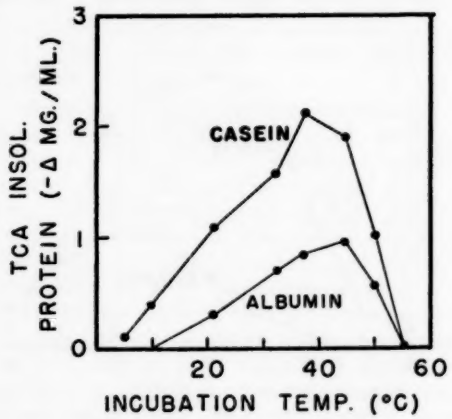

Fig. 4 Effect of incubation temperature on the proteolysis of casein and albumin (1 ml. of enzyme preparation incubated with substrate at pH 7.0 for 3 hours).

0.01 and 0.001 *M* concentrations were incubated with 1 ml. of the cell-free medium at 37.5° C. for 1 hour prior to addition of the substrates casein or lactalbumin. The proteolytic activity was determined at 37.5° C. and pH 7.0 after incubation for 3 hours. None of the reducing agents affected the proteolytic activity to any appreciable extent.

One-ml. quantities of the following salts, $Ca(C_3H_5O_3)_2 \cdot 5H_2O$, $Fe_2(SO_4)_3$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, Cu-

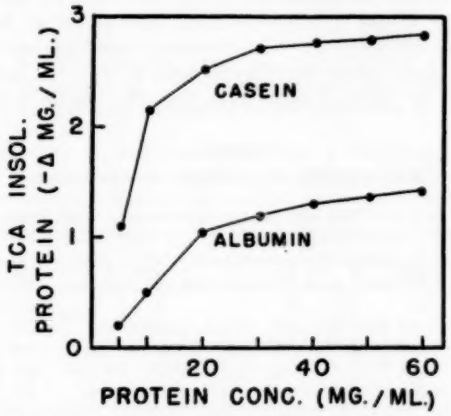

Fig. 5. Effect of substrate concentration on the proteolysis of casein and albumin (1 ml. of enzyme preparation incubated with substrate at pH 7.0 and 37.5° C. for 3 hours).

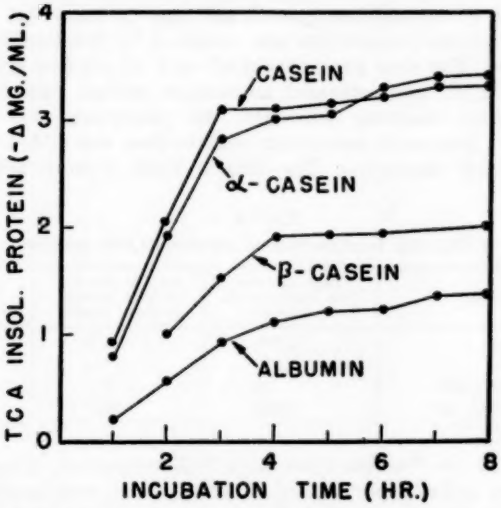

Figure 6. Effect of incubation time on the proteolysis of casein, α- and β-casein, and albumin (1 ml. of enzyme preparation incubated with substrate at pH 7.0 and 37.5° C.).

$SO_4 \cdot 5H_2O$, and $CoSO_4 \cdot 7H_2O$ in 0.01 and 0.001 *M* concentrations were added to 1 ml. of cell-free growth medium and incubated for 1 hour at 37.5° C. The mixtures then were incubated with casein and lactalbumin substrates at 37.5° C. at pH 7.0 for 3 hours. The metallic ions did not offect the proteolytic activity to any extent. In most cases they were slightly inhibitory.

Heat stability of proteolytic activity. Portions of cell-free growth medium adjusted to pH values ranging from 5.0 to 9.0 were adjusted to the same volume with sterile distilled water. The enzyme preparations then were heated at 55° C. for 1, 5 and 10 min., and at 60° C. for 1 and 3 min. Following cooling in ice-water, 1-ml. portions were used to test for proteolytic activity against casein with incubation at 37.5° C. and pH 7.0 for 3 hours. The data in Table 2 show that the proteolytic activity was quite

TABLE 2

Heat stability of proteolytic enzyme system

pH of enzyme preparation	TCA insol. protein (—Δmg./ml.)[1] after heating at:				
	55° C.			60° C.	
	1 min.	5 min.	10 min.	1 min.	3 min.
5.0	0	0	0	0	0
6.0	0.5	0	0	0.4	0
7.0	1.8	0.6	0	0.8	0
8.0	1.5	0.3	0	0	0
9.0	0	0	0	0	0

[1] One casein substrate. Control at pH 7.0:—2.3 mg./ml.

heat-labile. Heating at 60° C. for 3 min. inactivated the preparation. Maximum stability at 55° and 60° was found at pH 7.0.

Purification of the cell-free growth medium. A somewhat concentrated and purified enzyme preparation was obtained by fractionation with ammonium sulfate. The clear supernatant of one l. of cell-free growth medium was cooled to 2° C. and saturated ammonium sulfate was added to 50% saturation. After standing overnight, the precipitate was collected by centrifugation. Saturated ammonium sulfate then was added to the supernatant up to 75% saturation. The data in Table 3 show that the activity

TABLE 3

$(NH_4)_2SO_4$ fractionation of cell-free growth medium

Preparation	Total activity TCA insol. casein (—Δmg.)	Activity per mg. enzyme protein (—Δmg. casein)	% recovery
Cell-free medium	2,400	0.78	100
Satd. $(NH_4)_2SO_4$	2,012	—	83.8
0-50% Satd. $(NH_4)_2SO_4$	0	0	0
50-75% Satd. $(NH_4)_2SO_4$	1,864	42.0	77.7

was recovered in the fraction from 50 to 75% saturation. The data also indicate that the activity per mg. of enzyme protein had increased at least 50-fold by this fractionation, indicating that a considerable amount of inactive material had been removed.

SUMMARY

An extracellular heat-labile proteolytic enzyme system was recovered from the growth medium of *Ps. putrefaciens*. The enzyme system showed optimum activity against casein, α- and β-casein, and lactalbumin, at pH values from 7.0 to 7.5. Certain characteristics of this enzyme system have been studied. This proteolytic activity was precipitated in the 50 to 75% saturated ammonium sulfate fraction.

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PROTEOLYTIC ENZYMES FROM *PSEUDOMONAS PUTREFACIENS*. II. CHARACTERISTICS OF AN ENDOCELLULAR PROTEOLYTIC ENZYME SYSTEM [a]

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(Manuscript received October 1, 1956)

A previous investigation *(7)* showed that the cell-free growth medium of a milk culture of *Ps. putrefaciens* contained a heat-labile proteolytic enzyme system which attacked casein and lactalbumin. Subsequent experiments failed to show any peptidase activity in the extracellular preparation. The endocellular extract, however, was active against various di- and tripeptides. The literature reveals many reports on microbial peptidases; few, however, have been studied in detail. More recently Dudani *(1)*, Zimmerman *(8)*, and van der Zant and Nelson *(6)* reported on the peptidases of some of the lactic streptococci. The optimum pH for activity usually was between pH 7.0 and 8.5. In many instances metallic ions were necessary for optimum activity of microbial peptidases. The present study was undertaken to investigate the peptidases of *Ps. putrefaciens*.

EXPERIMENTAL METHODS

The culture *Ps. putrefaciens* 12 was the same organism as used in a previous study *(7)*. The stock culture was handled as described in that paper. The culture was transfered daily for 3 transfers prior to each experiment in the culture medium employed in the trial. One percent inoculum was used with incubation at 25° C.

Preparation of cell-free extract. *Ps. putrefaciens* 12 was grown in a medium of the following composition: 1 g. glucose, 5 g. yeast extract, 15 g. casamino acids, and 1 l. water. The above medium was autoclaved at 15 lb. steam pressure for 15 min., cooled to 25° C., adjusted to pH 7.0, and inoculated. The inoculated medium was incubated for 2 days at 25° C. The cells were collected by centrifugation in a Sharpless super centrifuge at 30,000 r.p.m. The cells were washed twice in ice cold phosphate buffer (pH 7.0, *M*/15) and collected by centrifugation in a refrigerated centrifuge and stored in a freezer unit. Cell-free extracts were prepared by disintegration of the cells by sonic vibration. A detailed procedure of this process was presented in a previous publication *(5)*. The cell-free extract was stored in a freezer unit and used within a few days.

Enzyme-substrate mixture. Buffered *M*/30 solutions of glycyl-L-tyrosine, L-leucyl-L-tyrosine, glycylglycine, glycylglycylglycine, glycyl-DL-valine, glycyl-DL-phenylalanine, glycyl-L-tryptophan, and *M*/15 solutions of DL-alanyl-DL-alanine, DL-alanylglycine, DL-alanyl-DL-asparagine, and DL-leucylglycine were used as substrates. The substrates were prepared as reported in a previous publication *(6)*. Hydrolysis of the peptides was determined by titration of the carboxyl groups with ethanolic KOH with thymolphthalein as indicator *(6)*. Unless stated otherwise, 0.2 ml. of cell-free extract was added to 3 ml. of substrate and incubated at pH 7.0 and 35° for 2 hours.

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RESULTS AND DISCUSSION

In Figure 1 are presented data showing the effect of time of incubation on the hydrolysis of nine dipeptides and one tripeptide by a cell-free ex-

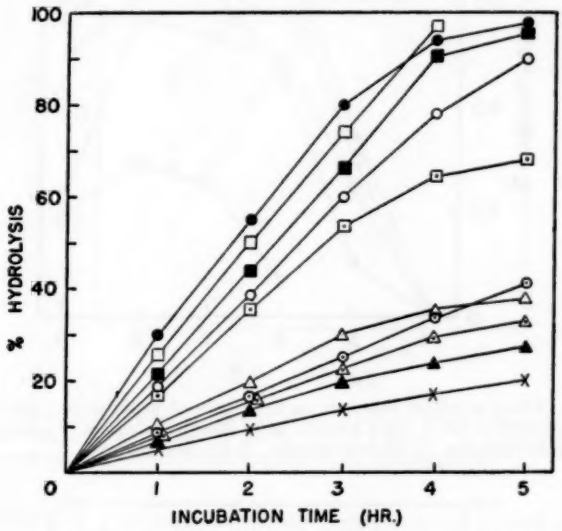

Figure 1. Effect of time of incubation on hydrolysis of peptides (0.2 ml. enzyme incubated with 3 ml. substrate at 35° C. and pH 7.0 for 2 hours). ● DL-alanyl-DL-alanine, □ L-leucyl-L-tyrosine, ■ glycyl-DL-phenylalanine, ○ DL-alanylglycine, ⊡ glycyl-DL-valine, △ DL-leucylglycine, ⊙ glycyl-L-tyrosine, ◬ glycylglycylglycine, ▲ DL-alanyl-DL-asparagine, × glycylglycine.

tract of *Ps. putrefaciens* 12 grown in a casamino acid broth. In most cases, a direct relationship was noted between time of incubation and peptide hydrolysis at least up to 3 hours of incubation. No activity was observed against glycyl-L-tryptophan. It is interesting to note that a cell-free extract of *Ps. putrefaciens* was able to hydrolyze the peptides containing the amino acid tyrosine. Previous studies have shown that various proteolytic enzyme systems such as pancreatic extract *(2)*, cell-free extract of *S. lactis (4)*, and pepsin and trypsin *(3)*, when acting on milk protein, cause a rapid increase in free tyrosine during the early phase of hydrolysis.

Figure 2 presents data showing the effect of pH of substrate on the hydrolysis of 5 dipeptides. Optimum hydrolysis was observed between pH 7.0 and 8.0. A sharp decrease in activity occurred at pH values below 6.0. The same was true for the activity against DL-alanylglycine at pH 9.0. As shown in Figure 3, optimum activity for most of the peptidases, within the temperature limits of this trial, was observed at 45° C.: for the peptidase active against L-leucyl-L-tyrosine at 35° C. A decrease in rate of hydrolysis with increasing temperature was noted for L-leucyl-L-tyrosine at 25° C., and for DL-alanyl-DL-alanine and glycyl-DL-phenylalanine at 35° C.

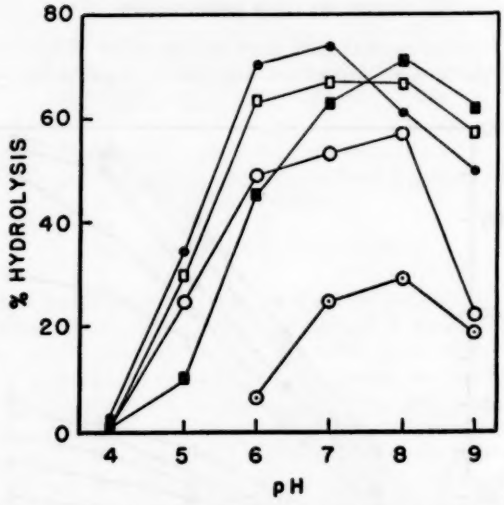

Figure 2. Effect of pH of substrate on hydrolysis of peptides (0.2 ml. enzyme incubated with substrate at 35° C. for 2 hours). ● DL-alanyl-DL-alanine, □ L-leucyl-L-tyrosine, ■ glycyl-DL-phenylalanine, ○ DL-alanylglycine, ⊙ glycyl-L-tyrosine.

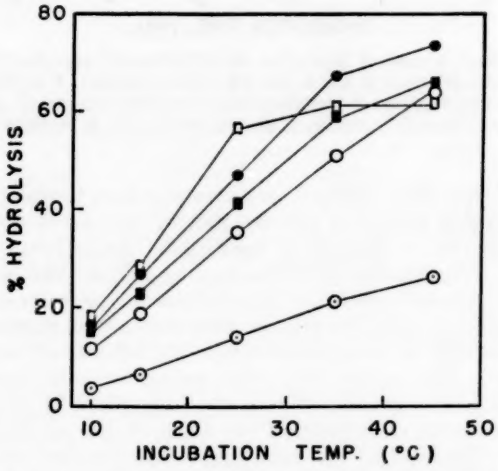

Figure 3. Effect of incubation temperature on hydrolysis of peptides (0.2 ml. enzyme incubated with substrate at pH 7.0 for 2 hours). ● DL-alanyl-DL-alanine, □ L-leucyl-L-tyrosine, ■ glycyl-DL-phenylalanine, ○ DL-alanylglycine, ⊙ glycyl-L-tyrosine.

Data in Figure 4 indicate a linear relationship between quantity of enzyme preparation and hydrolysis of most peptides up to 0.2 ml. of enzyme preparation; for the activity against glycyl-DL-phenylalanine up to 0.1 ml. of enzyme preparation.

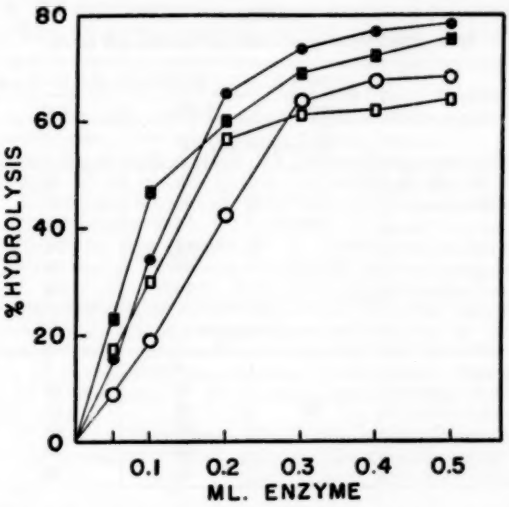

Figure 4. Effect of quantity of enzyme preparation on hydrolysis of peptides (enzyme incubated with substrate at pH 7.0 and 35° C. for 2 hours). ● DL-alanyl-DL-alanine, □ L-leucyl-L-tyrosine, ■ glycyl-DL-phenylalanine, ○ DL-alanylglycine.

Heat stability of peptidase activities. Portions of a cell-free extract of *Ps. putrefaciens* 12 were adjusted to pH values ranging from 4.0 to 9.0. Sterile distilled water then was added to adjust these preparations to the same volume. The extracts then were heated at 60° C. for 1, 4, and 8 min. and tested against DL-alanyl-DL-alanine, DL-alanylglycine, and glycyl-L-tyrosine as substrates with incubation at 35° C. and pH 7.0 (Table 1). At pH 7.0, little or no decrease in activity was observed even after heating for 8 min. At pH values of 5.0, and 9.0 a considerable decrease in peptidase activities took place when the cell-free extracts were heated for 4 min. or longer. No activity was observed at pH 4.0.

Effect of metallic ions on peptidase activities. Cell-free extracts (0.2 ml.) were incubated with 0.5 ml of metallic ion solution for 1 hour at 35° C. Following incubation the hydrolysis of DL-alanyl-DL-alanine, and DL-alanylglycine was determined after incubation for 3 hours at 35° C. and pH 7.0. The following solutions were used in 0.01, 0.001, and 0.0001 *M* concentrations. $MnSO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $NiCl_2$, and $CoSO_4 \cdot 7H_2O$. As shown in Table 2, addition of Mn and Mg increased the activity against DL-alanyl-DL-alanine, Cu, Zn, and Ni were inhibitory. Co did not show very much effect. With DL-alanylglycine as substrate, Mn increased the activity. Mg and Co did not show any effect; Cu, Zn, and Ni were inhibitory.

SUMMARY

A cell-free extract from *Ps. putrefaciens* 12 showed the presence of several peptidases with optimum activity between pH 7.0 and 8.0. Maximum

TABLE 1

Heat stability of peptidases at various pH levels [1]

pH of cell-free extract	% hydrolysis control	% hydrolysis after heating at 60° C. for:		
		1 min.	4 min.	8 min.
		DL-alanyl-DL-alanine		
4	80	0	0	0
5		60	40	25
6		78	60	45
7		82	78	80
8		70	55	30
9		70	24	0
		DL-alanylglycine		
4	65	0	0	0
5		51	45	30
6		55	52	50
7		68	66	70
8		68	60	56
9		58	38	0
		Glycyl-L-tyrosine		
4	50	0	0	0
5		40	32	20
6		48	40	42
7		48	46	50
8		45	30	25
9		38	28	0

[1] 0.2 ml. of enzyme preparation incubated with 3 ml. substrate at pH 7.0 and 35° C. for 2 hours.

TABLE 2

Effect of adding metallic ions on the peptidase activity [1]

Metallic ion added	% hydrolysis with metallic ion added in conc. of:		
	10^{-2} *M*	10^{-3} *M*	10^{-4} *M*
	DL-alanyl-DL-alanine		
None	60		
Mn	78	70	66
Cu	28	30	38
Zn	18	30	36
Mg	76	74	62
Ni	15	30	36
Co	56	60	56
	DL-alanylglycine		
None	52		
Mn	75	65	62
Cu	10	25	30
Zn	26	32	40
Mg	48	54	46
Ni	25	40	46
Co	50	48	52

[1] 0.2 ml. of enzyme preparation incubated with 3 ml. substrate at pH 7.0 and 35° C. for 2 hours.

heat-stability of the pepidase activity was observed at pH values near neutrality. The effect of several cations on the rate of hydrolysis was also studied.

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EFFECT OF THE PLATING MEDIUM ON THE SURVIVAL OF HEAT-TREATED CELLS OF *PSEUDOMONAS FLUORESCENS*[a]

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Previous investigations (*2, 3, 6,*) have shown that the characteristics of the growth medium used in studying survival of bacterial cells treated with heat or irradiation can have a pronounced effect on the number of survivors. Nelson (*3*) reported that certain heat-treated bacteria are more fastidious than others in their nutritional requirements. More recently, the same investigator (*4*) showed that the pH of the plating medium most favorable for growth of certain heat-treated organisms was not the same as for non-heated cells.

Some practical aspects of these observations deserve attention. The number of viable bacteria in heat-processed foods can be determined only by an indirect method such as colony formation, which represents reproductive capacity. The possibility exists then that under one set of conditions in the plating medium some of the heat-treated bacteria appear nonviable because of lack of growth but, when different conditions are available, may prove to be viable.

Although many investigations have shown that certain heat-treated bacteria grow better on the more complex rather than on simple media, few have shown a detailed study of the factors responsible for this phenomenon.

The present study was initiated to determine some of the nutritional factors in the plating medium which can affect the number of survivors of heat-treated cells of *Ps. fluorescens.*

EXPERIMENTAL METHODS

Culture. *Pseudomonas fluorescens* 29 was used in all experiments. This organism was isolated from milk and identified in this laboratory. For the individual trials, *Ps. fluorescens* 29 was grown in a basal medium of the following composition: 1 g. of K_2HPO_4, 5 g. of $NH_4H_2PO_4$, 5 g. of glucose, and 1 l. of distilled water. The medium was adjusted to pH 7.0 and autoclaved for 15 min. at 15 lb. steam pressure. Following inoculation, the culture was incubated at 25° C. for 24 hours. One per cent inoculum was used in all cases.

Heating trials. One ml. of a 24-hour culture of *Ps. fluorescens* 29 in basal medium was added to 99 ml. of hot basal medium (pH 7.0), which had been adjusted to the desired temperature in a constant temperature water bath. The suspension was agitated continuously by a stirrer. At certain intervals samples were taken and cooled immediately in sterile screw-cap test tubes submerged in ice water.

Plating media. Aliquots of the heat-treated and control (non-heated) suspensions were surface-plated on various solid media. The following media were employed: (a) nutrient agar (Bacto), (b) synthetic agar which contained the same ingredients as the basal medium with 15 g. of agar per l., (c) synthetic agar supplemented with 0.1% yeast extract, Bacto-peptone, beef extract, or casamino acids, (d) synthetic agar supplemented with amino acids, purines and pyrimidines, and vitamins. The various ma-

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terials which were added to the synthetic agar were sterilized by filtration through Seitz filters and were added just before pouring of the plates. The plates were incubated at 25° C. for 3 days.

RESULTS

Preliminary experiments (*1*) indicated that certain strains of *Ps. fluorescens*, including strain 29, and *Ps. fragi* grew equally as well on the simple synthetic agar as on nutrient agar. After 48 hours of incubation at 25° C., very little if any difference was noted in the size and number of colonies on the two media. On the other hand, heat-treated organisms did not grow as well on the synthetic agar as on nutrient agar and the colonies usually were somewhat smaller. The colony characteristics after 72 hours of incubation were similar to those after 48 hours of incubation, for both heated and non-heated suspensions.

In Figure 1 are presented representative data showing the number of survivors on 6 different plating media after heating at 55° C. for different lengths of time. The plate count of the non-heated control on the 6 different media was essentially the same. However, significant differences in the number of surviving heat-treated organisms were noted on the various plating media. The highest count of surviving organisms was observed on nutrient agar and synthetic agar with added yeast extract; the lowest count on synthetic agar. The counts on the synthetic agar with added beef extract, peptone, or casamino acids were intermediate between those on synthetic agar and nutrient agar.

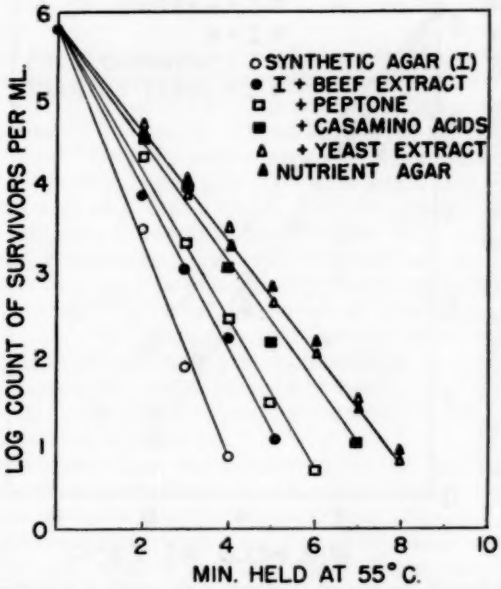

Figure 1. Effect of six different plating media on the number of surviving heat-treated Ps. fluorescens.

Incubation of the heat-treated cells on the plates with synthetic agar with or without supplement for more than 3 days did not increase the number of colonies or change the colony characteristics.

Subsequent experiments showed that surviving heat-treated organisms isolated from plates with synthetic agar plus supplement grew equally as well on synthetic agar as on synthetic agar plus supplement, or nutrient agar. The previous trials indicate that cells subjected to heat treatment are more demanding in their growth requirements. Apparently some factor(s) present in beef, yeast extract, peptone, or casamino acids enables the heat-treated cells to express themselves in greater numbers on the synthetic agar.

An attempt was made to prepare a synthetic medium which would substitute for the synthetic agar with added yeast extract. Stapleton *et al.* (*6*) have shown that a modified Lactobacillus assay medium gave approximately the same activity as yeast or spleen extract, in promoting recovery of irradiated cells of *Escherichia coli*. A synthetic medium, very similar to that of Stapleton *et al.* (*6*), gave results comparable to those obtained with synthetic agar with added casamino acids; however, it was not as effective as synthetic agar plus yeast extract (Figure 2). The composition of this medium is presented in Table 1.

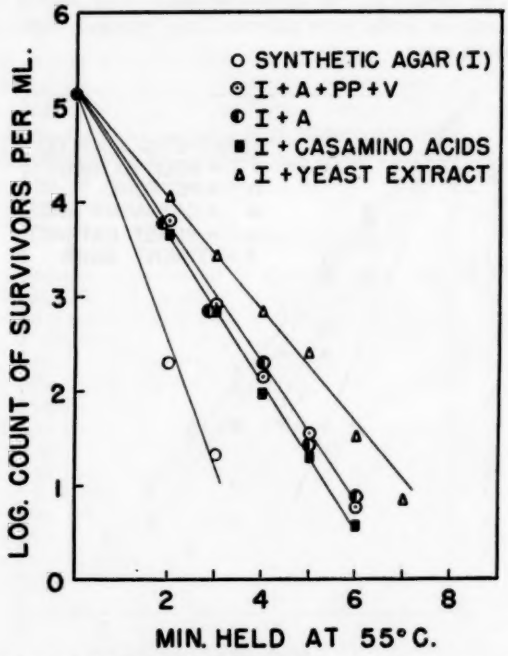

Figure 2. Effect of the various fractions of the modified Lactobacillus assay medium on survival of heat-treated cells of Ps. fluorescens. (A=amino acids, PP=purine-pyrimidine supplement, V=vitamins, see Table 1).

TABLE 1

Composition of modified Lactobacillus assay medium

DL-alanine	8 mg.	Adenine sulfate	10 mg.
L-arginine · HCl	4	Guanine · HCl	10
DL-aspartic acid	8	Uracil	10
L-cysteine · HCl	4		
L-glutamic acid	4	B_{12}	15 μg.
Glycine	4	Biotin	25
DL-histidine	8	Folinic acid	10
DL-isoleucine	8	Inositol	1 mg.
DL-leucine	8	Nicotinic acid	0.2
DL-lysine	8	p-aminobenzoic acid	10 μg.
DL-methionine	8	Pyridoxine · HCl	2 mg.
DL-phenylalanine	8	Riboflavin	0.2
L-proline	4	Thiamine · HCl	0.2
DL-serine	8		
DL-threonine	8	K_2HPO_4	1 g.
DL-tryptophan	8	$NH_4H_2PO_4$	5
L-tyrosine	4	Glucose	5
DL-valine	8	Agar	15
	water 1,000 ml.		

The data in Figure 2 indicates that the amino acid supplement was responsible for the counts of surviving heat-treated organisms on the modified Lactobacillus assay medium being higher than on synthetic agar. Counts of surviving heat-treated organisms on synthetic agar, and synthetic agar with the purine-pyrimidine-, or vitamin supplement were nearly the same.

An attempt was made to determine which amino acid or combination of amino acids was responsible for the greater number of survivors of heat-treated organisms on the synthetic agar with 18 amino acids.

Ps. fluorescens 29 was heated in basal medium at 55° C. Samples were taken at one-minute intervals over a period of 9 min. and plated on (a) synthetic agar, synthetic agar plus 18 amino acids, and synthetic agar plus one amino acid at a time (Figure 3A), (b) synthetic agar with 17 amino acids, eliminating one at a time (Figure 3B), and (c) synthetic agar with added glutamic acid plus one amino acid at a time (Figure 3C). The amino acids were used in concentrations as shown in Table 1.

The counts of the non-heated suspension on the different media were essentially the same. The semilogarithmic survival curve is a straight line and actual counts need not be reported. The effect of individual or combinations of amino acids on the survival of heat-treated organisms is expressed in terms of the length of heating required to reduce the count of the heated suspension of 0 per ml.

When the synthetic agar was supplemented with one amino acid at a time, only the medium with glutamic acid gave a slightly higher count for the heat-treated survivors than the synthetic agar (Figure 3A). The omission of a single amino acid from a mixture of 18 added to synthetic agar showed that the elimination of glycine, histidine, lysine, methionine, or proline resulted in lower counts of heat-treated survivors than with all 18 amino acids present (Figure 3B). The same was true, although not as pronounced, with respect to cysteine and glutamic acid.

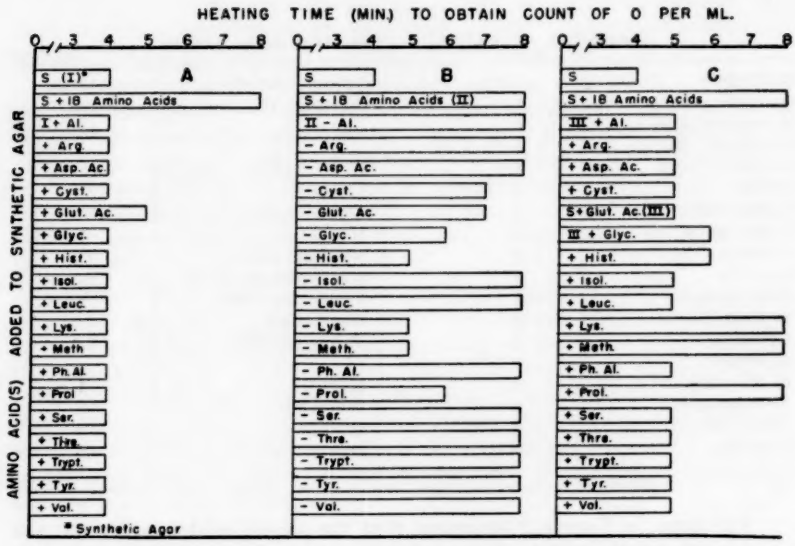

Figure 3. Effect of amino acids on the survival of heat-treated Ps. fluorescens.

Subsequent experiments in which single amino acids were added to synthetic agar supplemented with glutamic acid (Figure 3C) showed that a combination of glutamic acid with either lysine, methionine, or proline gave counts of heat-treated survivors similar to those with all 18 amino acids present. The additions of glycine or histidine to the synthetic agar plus glutamic acid increased the count of heat-treated survivors somewhat. No effect was noted with the other amino acids.

DISCUSSION

The present study with *Ps. fluorescens,* as well as that of Nelson (*3*) with certain other organisms, indicates that the counts of surviving heat-treated organisms are higher on more complex media, such as nutrient agar or yeast extract agar, than on simple synthetic media.

Although this observation usually is explained as a heat-induced requirement for nutritional factors, the exact nature of this phenomenon is unknown. There are several possibilities. Inactivation of an enzyme system(s) in some of the bacteria may induce a requirement for certain intermediates which are present in the complex media. However, the possibility exists that certain factors present in the complex media contribute to synthetic processes (protein synthesis, for example) which help repair the destructive action of heat. If certain systems vital for reproductive activities can be activated on the more complex media, a greater number of survivors can be expected.

The present study indicates that glutamic acid, glycine, histidine, lysine, methionine, and proline are involved in some manner. The activity of glutamic acid is not surprising in the light of the central physiological

role of this amino acid. It is interesting to note that glutamic acid plays an important role in "promoting recovery" of cells of *E. coli* injured by irradiation (*6*). The same was true for purine-pyrimidine supplements. The latter observation, however, could not be substantiated in this study.

The role of the other amino acids in "promoting recovery" of heat-treated cells is not as clear and only certain speculations can be made. It has been shown (*8*) that in *E. coli* glutamic acid is the precursor of proline and ornithine and other metabolic links between proline and ornithine have been found for *E. coli, N. crassa,* and *T. utilis.*

Histidine-adapted cells of *Ps. fluorescens* (*7*) can convert histidine to L-glutamic acid, formic acid, and ammonia. In *Neurospora,* synthesis of lysine proceeds by amination of α-aminoadipic acid; in *E. coli,* by decarboxylation of diaminopimelic acid (*9*). The role of methionine in the present studies would be difficult to ascertain, because of the great number of metabolic reactions in which it is involved. The same is true for glycine. The α-carbon atom is utilized for the synthesis of porphyrins, ureido groups of purines, serine, and methyl groups (*5*).

Although many of the metabolic links and important cellular functions of these amino acids have been studied in detail, very little is known about the role of these amino acids in "promoting recovery" of heat-injured cells. It is hoped for that further studies will elucidate their role in this process.

SUMMARY

A greater number of heat-treated survivors of *Ps. fluorescens* were found on complex media, such as nutrient agar and yeast extract agar, than on simple synthetic media. The amino acids glutamic acid, proline, histidine, glycine, and methionine are in some, at the present unknown, way involved in a process which enables heat-treated cells to express themselves in greater number on the simple synthetic media.

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CHANGES IN THE BIACETYL CONTENT OF CREAMED COTTAGE CHEESE CAUSED BY ORGANISMS ASSOCIATED WITH SPOILAGE

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(Manuscript received October 11, 1956)

Biacetyl produced during the fermentation of citric acid by *Leuconostoc citrovorum* and/or *Leuconostoc dextranicum* is primarily responsible for some of the desirable flavor and aroma in dairy products, including cottage cheese. Acetylmethylcarbinol, produced by the same fermentation scheme under more anaerobic conditions, is readily oxidized to biacetyl. Krishnaswamy and Babel (*5*) found 1.06 to 2.55 p.p.m. of biacetyl in dry cottage cheese curd made by the long-set method. Elliker (*3*) observed that *Streptococcus lactis* had no effect on biacetyl in butter, but that several species of *Pseudomonas* destroyed biacetyl. Parker and Elliker (*6*) reported rapid decreases in biacetyl and losses of aroma in cottage cheese inoculated with cultures of *Pseudomonas viscosa* and *Pseudomonas fragi;* whereas, *Alcaligenes metacaligenes* was less active in biacetyl destruction. They reported the biacetyl was converted to acetylmethylcarbinol and possibly 2, 3-butylene glycol.

Twenty-four different organisms capable of causing spoilage in cottage cheese were isolated and identified by Bonner (*1*). These organisms represented 7 genera of bacteria, 3 genera of molds, and 2 genera of yeast. Varying degrees of flat flavor were encountered in cheese inoculated with these organisms and stored at 40° and 50° F. An investigation of methods of increasing biacetyl in cottage cheese suggested the desirability of determining the amounts of biacetyl usually occurring in creamed cottage cheese, and the effect of several different organisms on the biacetyl and acetylmethylcarbinol content of cottage cheese stored at 40° and 50° F.

EXPERIMENTAL PROCEDURE

Thirty samples of commercial fresh creamed cottage cheese secured from processing plants or retail markets were examined organoleptically and analyzed for biacetyl and acetylmethylcarbinol content.

Twelve organisms representing bacteria, yeast, and molds capable of causing spoilage were inoculated into cottage cheese. The organisms were *Geotrichum candidum, Mucor plumbeus, Rhodotorula flava, Pseudomonas fluorescens, Pseudomonas tralucida, Ps. fragi, Achromobacter butyri, Achromobacter eurydice, Alcaligenes metalcaligenes, Bacillus firmus, Micrococcus flavus,* and *Escherichia coli.*

One lot of cottage cheese curd was divided into 13 equal portions. An appropriate amount of creaming mixture containing 14% butterfat and 4% salt was also divided into 13 equal parts. One lot of cheese was creamed and used as a control. Each of the remaining lots of cream was inoculated with 0.1 ml. of a 24-hour trypticase soy broth culture of one of the above microorganisms. The inoculated lots of cream were added to the separate portions of curd and the creamed cheese was packaged into 12-oz. cartons.

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[b] Mich. State Univ. Agr. Expt. Sta., Journal Article No. 1961.

It is impossible to sterilize cottage cheese without resorting to procedures which alter the physical characteristics of the curd or interfere with the normal activity of subsequent inoculants. An attempt was made to introduce sufficient inoculation of the known contaminant to dominate the fermentation, and in each instance the spoilage was characteristic of the organism inoculated. The number of organisms added ranged from 300 molds per ml. in the cream inoculated with *Mucor plumbeus*, to 450,000 coliforms per ml. in the cream inoculated with *E. coli*. One-half of the packages from each lot was stored for 12 days at 40° F. and the other half stored for 6 days at 50° F. Spoilage rendered longer storage times impractical.

Biacetyl, acetylmethylcarbinol, pH, and organoleptic determinations were performed initially and at 3-day intervals on each sample of creamed cottage cheese. Biacetyl and acetylmethylcarbinol were determined by the Prill and Hammer method (*7*), using a 25-g. sample of cheese. Color intensity was measured on a Cenco-Sheard-Sanford photelometer. Duplicate determinations were frequently performed and agreed within 1 p.p.m. The pH was determined on thoroughly macerated cheese, with a Beckman Model G pH meter.

RESULTS AND DISCUSSION

Amount of biacetyl and acetylmethylcarbinol in fresh creamed cottage cheese. Biacetyl contents of 30 samples of commercial fresh creamed cottage cheese ranged from 0.7 to 10.4 p.p.m., with most samples containing from 3 to 8 p.p.m. A flat flavor was usually apparent in samples containing less than 3 p.p.m. The amount of acetylmethylcarbinol varied from 15 to 50 p.p.m. with most samples containing from 32 to 38 p.p.m. Hammer (*4*) reported that butter usually contained about ten times more acetylmethylcarbinol than biacetyl.

Effect of various organisms on the biacetyl content of creamed cottage cheese. Data for samples stored 12 days at 40° F. and 6 days at 50° F. are shown in Figures 1 to 4 and 5 to 8, respectively. Most of the organisms used in this work caused a reduction in the biacetyl content of cottage cheese stored at 50° F. In the samples held at 40° F., most of the organisms caused very slight reductions in biacetyl during the first 6 days and only moderate decreases thereafter.

The biacetyl content of all the cheese at 0 day was 6.4 p.p.m. In the control sample stored at 40° F. there was a gradual increase in biacetyl to a maximum of 9.4 p.p.m. on the 9th day, after which there was a moderate decrease to 8.5 p.p.m. on the 12th day. A slight decrease between the 3rd and 6th day was within the range of experimental error. In the control sample stored at 50° F. the biacetyl value increased to 10.2 p.p.m. on the 3rd day and decreased to 4.6 p.p.m. on the 6th day. These results agree in general with those of Krishnaswamy and Babel (*5*), who observed an increase in the biacetyl content after 4 days and a decrease after 7 days, when good quality dry curd was stored at 40° F.

Ach. butyri is the only organism which caused an increase in biacetyl at both storage temperatures (Figures 3 and 7). At 40° F. the biacetyl content of the cheese inoculated with *Ach. butyri* increased continuously throughout the holding period, exceeding the control on the 6th day and thereafter. The cheese inoculated with *Ach. butyri* was the only sample in which the biacetyl content increased on the 3rd day, although the value was below the corresponding control.

The *Pseudomonas* organisms caused greater decreases in biacetyl at 40° F. than any other organisms studied (Figure 2). However, at 50° F.

Effect of microorganisms on the biacetyl content of creamed cottage cheese stored 12 days at 40° F.

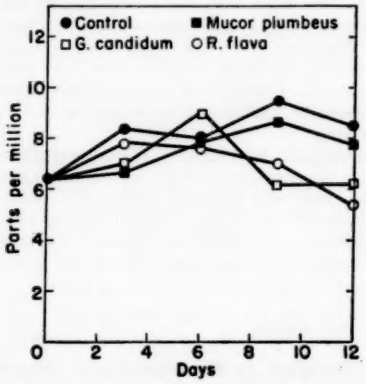

Figure 1. Variations in the biacetyl content of creamed cottage cheese inoculated with G. candidum, Mucor plumbeus, or R. flava.

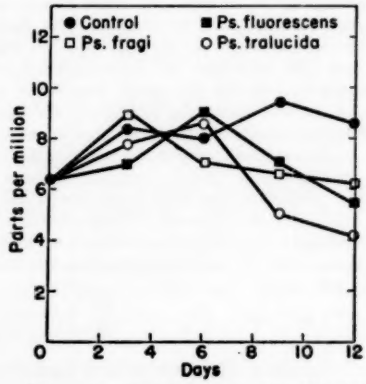

Figure 2. Variations in the biacetyl content of creamed cottage cheese inoculated with Ps. fragi, Ps. fluorescens, or Ps. tralucida.

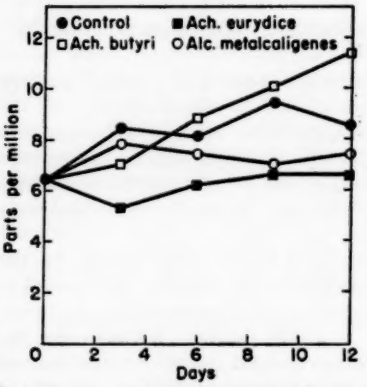

Figure 3. Variations in the biacetyl content of creamed cottage cheese inoculated with Ach. butyri, Ach. eurydice, or Alc. metalcaligenes.

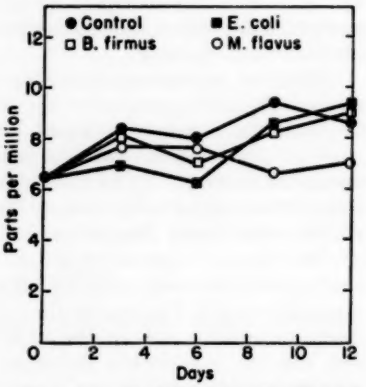

Figure 4. Variations in the biacetyl content of creamed cottage cheese inoculated with B. firmus, E. coli, or M. flavus.

(Figure 6) these decreases were equalled or excelled by the yeast and molds. *Alc. metalcaligenes* caused only a slight decrease in biacetyl at both 40° and 50° F. (Figures 3 and 7). These results agree with those of Parker and Elliker (*6*), except these workers observed greater destruction of biacetyl at 50° F. by several strains of *Pseudomonas* than encountered in the work reported herein.

At both 40° and 50° F. the biacetyl content of the cheese inoculated with *M. flavus* and *B. firmus* (Figures 4 and 8) remained relatively constant, with the terminal values being slightly greater than the control.

Effect of mircoorganisms on the biacetyl content of creamed cottage cheese stored 6 days at 50° F.

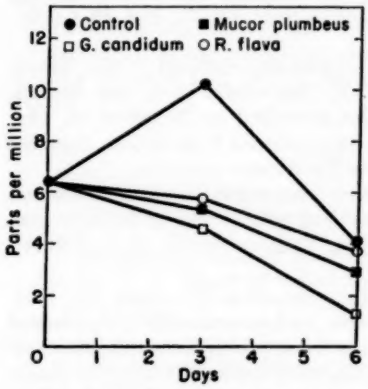

Figure 5. Variations in the biacetyl content of creamed cottage cheese inoculated with G. candidum, Mucor plumbeus, or R. flava.

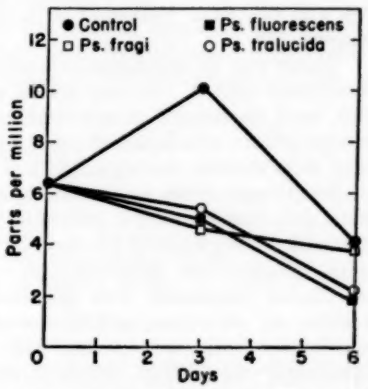

Figure 6. Variations in the biacetyl content of creamed cottage cheese inoculated with Ps. fragi, Ps. fluorescens, or Ps. tralucida.

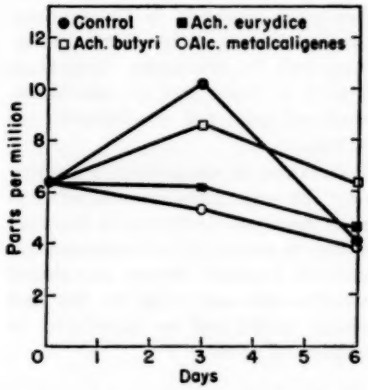

Figure 7. Variations in the biacetyl content of creamed cottage cheese inoculated with Ach. butyri, Ach. eurydice, or R. flava.

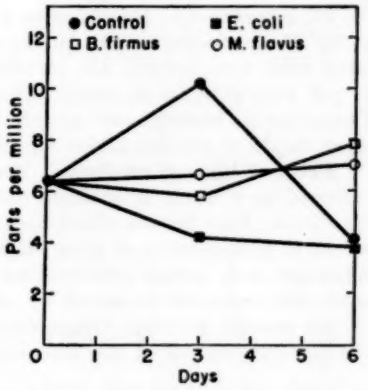

Figure 8. Variations in the biacetyl content of creamed cottage cheese inoculated with B. firmus, E. coli, or M. flavus.

Biacetyl was not completely destroyed in any sample. Cheese containing definite slime formation contained biacetyl in amounts ranging from 0.4 to 8.0 p.p.m.

Effect of various organisms on the acetylmethylcarbinol content of creamed cottage cheese. The acetylmethylcarbinol content of the control sample of creamed cottage cheese stored at 40° F. was 45, 50, and 40 p.p.m. at 0, 6, and 12 days, respectively. None of the organisms caused significant change in the acetylmethylcarbinol content of the cheese stored at 40° F.

In the control cheese stored at 50° F. there was a decrease in acetylmethylcarbinol during the first 3 days and a substantial increase between the 3rd and 6th day. Values at 0, 3, and 6 days were 45, 25, and 49 p.p.m., respectively.

There was an enormous increase in acetylmethylcarbinol in the cheese inoculated with *E. coli* and stored at 50° F. The strain of *E. coli* used in this work conformed to the characteristics described by Breed *et al.* (*2*), except gelatin was liquefied and no acid was produced from xylose. Laboratory tests showed the organism was Voges-Proskauer—negative.

Smaller increases in aceylmethylcarbinol occurred in samples inoculated with *Ach. butyri* and *Ps. tralucida*, and held at 50° F. The latter organism caused a corresponding decrease in biacetyl. Similar results were observed with *Pseudomonas desmolyticum*.

Cheese inoculated with *Rh. flava*, *Ach. eurydice*, *Ps. fragi*, *Ps. fluorescens*, *G. candidum*, and *Mucor plumbeus*, and stored at 50° F., showed continuous uniform decreases with virtual disappearance of aceylmethylcarbinol at the 6th day. The acetylmethylcarbinol content of samples inoculated with *B. firmus*, *M. flavus*, and *Alc. metalcaligenes* conformed closely to the control throughout the 6-day storage interval.

Effect of pH on changes in biacetyl and acetylmethylcarbinol. The pH value of all samples of creamed cottage cheese was 4.85 at 0 days and varied from 5.4 to 4.5 and from 5.2 to 4.5 among the samples stored at 40° and 50° F., respectively. Decreases in pH were more frequent in the samples at 50° F. Increases in pH during storage were greatest in cheese inoculated with *Ach. butyri*, *Alc. metalcaligenes*, and *Ps. tralucida*. Decreases in pH were greatest in cheese inoculated with *R. flava* and *G. candidum*. Variations in biacetyl and acetylmethylcarbinol were not consistently related to pH of the inoculated samples studied.

Effect of biacetyl on flavor. Most deterioration in organoleptic quality occurred as a result of spoilage induced by the organisms inoculated into the cheese. Flat flavors which occurred as a result of decreases in biacetyl were less prominent than other off-flavors, due to action of microorganisms. Although *Ach. butyri* produced an increase in biacetyl, cheese inoculated with this organism possessed an objectionable aromatic odor at the end of the storage periods. Organoleptic quality could not be correlated to the biacetyl content of the inoculated samples of cheese.

SUMMARY

Biacetyl content of fresh creamed cottage cheese ranged from 0.7 to 10.4 p.p.m. The acetylmethylcarbinol content of the same samples varied from 15 to 50 p.p.m.

Several organisms commonly identified with spoilage in cottage cheese were individually inoculated into creamed cottage cheese which was stored at 50° and 40° F. for 6 and 12 days, respectively. The biacetyl content of the cheese inoculated with *Achromobacter butyri* increased during storage. The *Pseudomonas* organisms caused greater decreases in biacetyl than any other organisms studied.

No appreciable changes occurred in the acetylmethylcarbinol content of inoculated cheese stored at 40° F. Cheese inoculated with *Rhodotorula*

flava, Achromobacter eurydice, Pseudomonas fragi, Pseudomonas fluorescens, Geotrichum candidum, and *Mucor plumbeus,* and stored at 50° F., showed continuous decreases in acetylmethylcarbinol.

Variations in biacetyl and acetylmethylcarbinol were not consistently related to pH of the inoculated samples studied. Neither could organoleptic quality be correlated to the biacetyl content of the inoculated samples of cheese.

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MAPLE SIRUP. X. EFFECT OF CONTROLLED FERMENTATION OF MAPLE SAP ON THE COLOR AND FLAVOR OF MAPLE SIRUP [a]

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Maple sirups produced during the early periods of the maple season are characteristically light amber in color and delicately flavored; it is this type of sirup that is highly valued. As the season advances the sirup becomes progressively darker and acquires a stronger flavor which may not be entirely due to maple. These changes have been mainly associated with the growth of microorganisms in the sap from which the sirup is made *(2, 3, 4)*. The association of color of maple products with quality has been so emphatic that color has become the chief criterion for grading maple sirup.

Studies at this laboratory on the flavor of maple sirup have shown that the desirable flavor component can be increased several fold by heat treatment under special conditions. This indicated that maple sap contains flavor and color precursors which are modified during the processing of the sap to sirup. Although this modification is generally induced by heat during the processing procedure, it could also be influenced by microbiological activity in the sap. These studies were made to determine whether or not desirable changes in flavor and color of maple sirup can result from the fermentation of sap by individual species or combinations of various species of microorganisms. This work establishes that maple flavor can be intensified through proper fermentation of the sap.

MATERIALS, METHODS, AND PROCEDURE

Materials. Maple sap used in these studies was collected under aseptic conditions using the technique described in a previous publication *(4)*. Sap from each sterile 5-gallon collecting bottle was aseptically dispensed into sterile 1-gallon metal cans, quick-frozen, and stored at −29° C. Sterility of the collected sap was determined by sampling each collecting bottle and plating the samples in nutrient agar (7). Any lot of sap having a count exceeding one colony per ml. was discarded. Since all of the fermentations could not be conducted simultaneously, they were made at 4 different times with four lots of sap. Careful selection of sap was required so that identical replicates could be used throughout the 4 parts of the study. To obtain this uniformity, sixteen 4-gallon collections of sterile sap were selected. One gallon of sap from each collection was allocated to each of the 4 lots so that at the completion of the allocation each 16-gallon lot contained equal volumes of sap from the same trees and the same flows.

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[a] Presented at the 130th National Meeting of the American Chemical Society, September 16-21, 1956, Atlantic City, New Jersey.

[b] A laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

To prepare each lot of frozen sap for use in fermentation studies, a procedure was developed to thaw the sap rapidly in a manner which minimized microbial contamination and chemical changes. The desired amount of frozen sap was removed from the gallon cans, crushed in a sanitized power crusher and the crushed ice melted rapidly in a steam jacketed kettle. To prevent changes in the sap during heating, the kettle was charged with about one gallon of the crushed frozen sap, heated with steam at 5 p.s.i. and the ice slurried rapidly until the greatest portion had melted, at which time the sap was removed. The residual ice melted in a short time and the final temperature of the sap was 15-20° C. The melted sap was blended and dispensed into sterile 50-liter carboys for fermentation.

Cultures. Four strains of bacteria and one strain of yeast, all isolated from naturally fermenting sap, were used in these studies. All of the bacterial strains were aerobic, gram negative rods capable of developing good growth at temperatures of 2-3° C. Two of the cultures develop light cream colored colonies and produce a bluish-green water soluble pigment. The cells of these two cultures are very motile, having 3 to 5 flagella at one pole. They produce proteolysis of milk and liquefaction of gelatin. Preliminary taxonomic studies *(7)* place these two organisms in the genus *Pseudomonas*. One of these cultures is designated as *Pseudomonas*-11 and the other *Pseudomonas*-25. A third culture produces a yellow pigmented colony and the cells are non-flagellate. It produces proteolysis when cultured in milk and causes liquefaction of gelatin. This bacterium is tentatively classified as a species of *Flavobacterium* and is designated here as *Flavobacterium*-583. The fourth culture produces colonies with a pink-orange pigmentation. The cells are motile and possess a single polar flagellum The culture does not cause proteolysis of milk or liquefaction of gelatin and at the present time has not been classified but is designated as *Bacterium*-587.

Methods. Refractive index measurements for the determination of per cent solids as sucrose (degrees Brix) were made at 20° C. with the Abbe refractometer. Color indices[c] *(6)* were determined from absorption measurements at 450 mμ made on sirups of 65-67° Brix pressure filtered through three layers of Whatman[d] No. 52 filter paper in a Seitz filter. Per cent of invert sugar was determined by the Berlin method *(5)*. Bacterial counts were made by plating appropriate dilutions of sap samples on nutrient agar and yeast counts were made on Wickerham's Agar *(9)*. Plates were incubated at room temperature for 48 hours. Flavor evaluations of the sirups were made by an experienced panel.

Procedure. The study consisted of 8 separate fermentations, 5 being produced by specific strains of microorganisms and 3 by combinations of specific bacteria and a yeast. The collected sap for these fermentations was taken from frozen storage in 4 successive lots of 16 gallons each (as described above) and thawed. Two gallons of each lot were retained for conversion to the control sirup, and the remaining 14 gallons were divided and dispensed into two sterile carboys for inoculation. The procedure was repeated for the remaining 6 fermentations.

Inocula were prepared from actively growing cultures by washing the organisms from agar slants and making appropriate dilutions. Where combinations of organisms were used for inoculations, the washings from the selected slants were combined to prepare the inoculum. The number of organisms in the inoculum for each fermentation was determined from plate counts of sap samples taken immediately after inoculation.

The inoculated sap was incubated in a cold room at 0.5-2.5° C. which approximates temperature conditions occurring in the field during the sap season. At the end of 3, 6, and 10 days of incubation, samples were withdrawn from each inoculated carboy for plate counts and 2-gallon portions were removed aseptically for conversion to sirup.

The 2-gallon portions of sap were reduced to sirup using the following standardized conditions selected to simulate the conditions occurring in a commercial evaporator. Using a steam-jacketed kettle, the sap was evaporated rapidly (30-35 minutes) to a

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[c] Color index, $A^{65\text{-}67\%}_{1\,cm.} = A^{1}_{420}$ (86.3/bc), where A_{450} is the observed absorbance at 450 mμ, b is the depth of solution in centimeters, and c is the grams of solids as sucrose per 100 ml.

[d] Mention of commercial products does not imply endorsement by the Department of Agriculture over others of similar nature not mentioned.

volume calculated to give a density of approximately 45° Brix. This sirup was transferred to a stainless steel beaker equipped with a condenser and refluxed for one hour over a Meeker burner. The refluxed sirup was transferred to a 1-quart steam kettle and evaporated rapidly (5-8 minutes) to standard density sirup (65.5° Brix).

The following determinations were made on the sirups prepared from the fermented sap: per cent of invert sugar, color indices, and flavor evaluations.

RESULTS AND DISCUSSION

Table 1 shows the growth of microorganisms in maple sap. Increase in number of microorganisms is found in all cases except one where the combination of *Bacterium*-587 and yeast shows a decrease after the third day of incubation. The increased growth at these low temperatures establishes

TABLE 1

Growth of microorganisms inoculated into maple sap

Organisms	Number of microorganisms per ml. of sap			
	Days of incubation			
	0	3	6	10
Pseudomonas–25	1,090,000	18,300,000	lost	58,000,000
Bacterium–587	1,040,000	3,100,000	2,500,000	8,000,000
Yeast	31,000	390,000	1,580,000	4,600,000
Pseudomonas–11	1,440,000	lost	25,000,000	59,000,000
Flavobacterium–583	310,000	1,610,000	3,100,000	2,770,000
Pseudomonas–11 and Yeast	850,000	lost	5,500,000	52,000,000
Flavobacterium-583 and Yeast	330,000	1,400,000	7,400,000	12,200,000
Bacterium–587 and Yeast	4,300,000	8,500,000	5,100,000	3,700,000

that maple sap is a good growth medium for these adventitious organisms; this is especially noticeable in the case of the species of *Pseudomonas* where growth increased approximately 60 fold.

The changes in maple sirup produced from sap that had been fermented with 8 different inocula are presented in Table 2. These include development of flavor, color, and free reducing sugars. Flavors were of two types: a full-bodied maple flavor and an acrid caramel flavor. Levels of maple flavor are expressed on a scale of 4, with 1 corresponding to the flavor level present in a commercial U. S. grade AA maple sirup, and 2, 3, and 4 indicating increments of increasing intensities. In the case of caramel, 1 designates the lowest detectable level and levels of increasing intensities are designated by 2, 3, and 4 where 4 represents a strong caramel flavor.

With one exception, all of the sirups made from sap fermented by these organisms had a distinctive maple flavor with an intensity level of 1 or more. Fermentation of sap by *Pseudomonas*-25 produced the greatest effect. The flavor level attained a score of 2 after only 3 days of fermentation, and was increased to a score of 3 and 4 after 6 and 10 days, respectively. The unusual nature of this fermentation was the development of a high level of maple flavor without the production of caramel, although the color of the sirup was greatly increased. In commercial practice, the production of a dark colored sirup (U. S. unclassified) rich in maple flavor and free of caramel is never encountered. Another unusual characteristic of the sirup resulting from this fermentation was the lack of any residual

TABLE 2

Properties of maple sirup produced from sap fermented by various microorganisms

	Increase in color index over control			Change in per cent invert sugar over control			Flavor [1]					
							Maple			Caramel		
Days incubated	3	6	10	3	6	10	3	6	10	3	6	10
Organism:												
Pseudomonas–25	0.22	0.61	2.15	0.0	0.0	+0.1	2	3	4	0	0	0
Bacterium–587	0	0.19	0.28	0	lost	0.0	1	1	1	0	0	0
Yeast	0.03	0.43	1.78	0.0	+0.3	+5.2	1	1	1	0	1	4
Pseudomonas–11	0.35	0.47	2.11	+0.1	+0.1	+0.1	1	1	1	0	0	1
Flavobacterium–583	0.20	0.52	1.15	+0.0	+0.4	+0.6	2	2	2	0	1	2
Pseudomonas–11 and Yeast	0.45	0.81	2.78	+0.1	+0.1	+0.3	1	2	2	0	0	2
Flavobacterium–583 and Yeast	0.15	1.09	1.49	+0.1	+1.4	+2.2	2	2	2	0	2	4
Bacterium–587 and Yeast	0.09	0.29	1.07	+0.0	+0.2	+1.0	off-flavor, increasing with time					

[1] Flavor has been given numerical values of 0, 1, 2, 3, and 4 with 0 being lack of the particular flavor and 4 being the greatest amount. In the case of maple, 1 is indicative of good maple flavor as exhibited in the control sirup.

reducing sugars. In commerce all strongly flavored, dark-colored sirups (U. S. grade B or below) contain relatively large amounts of free reducing sugars.

Sap fermented with *Bacterium*-587, like that fermented by the *Pseudomonas*-25, yielded sirup that contained no caramel flavor and did not cause the formation of any free reducing sugars. This sap did not, however, yield a sirup of increased maple flavor even after the 10 days of fermentation. It should be noted that this organism did not develop a high population in this medium and its total population after 10 days incubation was only one-tenth that of *Pseudomonas*-25.

The effect on flavor of fermenting sap with *Pseudomonas*-11 was much the same as that caused by *Bacterium*-587 except that a trace of caramel flavor was detected after 10 days' incubation. The growth rate of this species of *Pseudomonas* was very similar to that of the other representative of this genus.

The sirup made from sap fermented by yeast showed the development of a dark color and a strong caramel flavor accompanied by a large increase in free reducing sugars. This is in agreement with current observations *(8)* which indicate that in heated sugar solutions the amount of color developed is related to the amount of reducing sugars present.

The flavor formed in sirup produced from sap fermented by *Flavobacterium*-583 was intermediate between that caused by *Pseudomonas*-25 and by yeast. The level of maple and caramel flavors was only one-half that caused by the *Pseudomonas* and yeast, respectively. The rate of maple flavor development in the case of *Flavobacterium* was more rapid since the sirup made from sap fermented 3 days had a maple flavor level of 2 as compared to a level of 1 for other fermentations with the exception of *Pseudomonas*-25. The *Flavobacterium* caused the formation of some reducing sugars. This could account for the production of caramel flavor in the sirup but not for the lighter color which was only one-half that formed

in the *Pseudomonas* fermentation. Failure of the *Flavobacterium* to cause the production of large amounts of maple and caramel flavors may be due to its slower growth rate in sap. Its population at the end of 10 days of incubation was only one-twentieth of that produced by the more rapidly growing *Pseudomonas* species.

In the natural uncontrolled fermentation of maple sap, it is doubtful that fermentation is ever caused by a pure culture. Therefore, fermentations using mixed cultures were conducted. For simplicity the mixtures included only two cultures. The yeast culture was used in every mixture because yeasts often appear as one of the predominating groups of organisms late in the season, and it was desired to learn the effect of this culture on flavor and color of sirup when grown in combination with bacteria.

In the yeast-*Pseudomonas*-11 mixture there was a slight increase in the amount of maple flavor produced after 6 days of incubation as compared to that produced by each organism separately, and the amount of caramel produced was diminished over that produced by yeast alone. This may indicate that the rapidly growing *Pseudomonas* retarded the growth of the yeast.

The effect of the mixed culture of *Flavobacterium*-583 and yeast was as would be expected. The slow growing *Flavobacterium* did not retard the growth of the yeast so that the net effect on the sirup was as if each were causing the fermentation independently, the *Flavobacterium* causing a maple flavor level of 2 and the yeast a caramel flavor level of 4.

The effect on flavor of the mixed culture of *Bacterium*-587 and yeast was unexpected. Even after only 3 days of fermentation it imparted to the sirup a pronounced off-flavor which increased with time. This off-flavor was not present in sirup made from sap fermented with either of these organisms separately.

The observation that fermentation of sap by certain microorganisms can induce changes which result in the intensification of the desired flavor may be a clue to the long sought explanation for the question: what makes maple flavor? In addition to these microbial fermentations the effect of culture extractives and enzymes is being investigated. An understanding of the changes which occur will be of great advantage in developing conditions for optimum processing of this product and these studies should ultimately lead to methods which will aid the control of flavor and color in maple sirup.

SUMMARY

This study shows that the fermentation of maple sap has a marked effect on the flavor and color of the sirup produced. Evidence is presented for generic differences in the degree and type of changes produced in the flavor and color. Some species of microorganisms produce intensification of maple flavor only, while others favor the production of caramel flavor or maple with varying degrees of caramel. The caramel flavor of maple sirup is correlated with the amount of free reducing sugars formed during microbial growth in sap, and is especially apparent in the case of yeast fermentation.

Acknowledgment

The authors wish to express their appreciation to R. A. Bell for his assistance during this study.

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AMINO ACID COMPOSITION OF BIRD'S NEST PROTEIN[a]

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"Swallow's nest soup," a famous dish in China, has long been considered to be a food of high nutritive value (*3*). The principal ingredients of this soup are to be found in the basic material used; namely, the nests built by birds of the swiftlet genus (*Collacalia*) which are indigenous to Indo-China and other parts of the Malaysian area (*4*). The nests are made of a white, translucent material similar to gelatine in appearance, and consist almost entirely of hardened saliva. It was deemed desirable to investigate the amino acid composition of birds' nests, in order to gain information on the nutritive value of this costly food.

EXPERIMENTAL

A sample of swallow's nest, purchased in Chinatown, Los Angeles, was ground to a fine powder. Nitrogen was determined by the Kjeldahl procedure and the ten essential amino acids by the microbiological assay methods of Dunn *et al.* (*5*, *6*). A 2-g. sample was prepared for amino acid analysis by hydrolyzing it for 18 hours with 50 ml. of 8 *N* HCl, filtering the hydrolysate, evaporating the filtrate to dryness under reduced pressure, dissolving the residual material in water, and diluting the solution to 100 ml. For the determination of tryptophan, a 2-g. sample was hydrolyzed by autoclaving it for 10 hours with 50 ml. of 5 *N* NaOH. An aliquot was neutralized and diluted to 100 ml. with distilled water.

RESULTS AND DISCUSSION

The percentages of the 10 essential amino acids in bird's nest and casein are given in Table 1. It may be noted that bird's nest is relatively rich in protein, that it compares favorably with casein in its percentages of

TABLE 1
Amino acid composition of bird's nest and casein[1]

Amino Acid	Bird's nest sample[2]		Average	Casein (7)
	A %	B %	%	%
Arginine	5.8	5.6	5.7	7.3
Histidine	2.9	3.0	3.0	3.1
Isoleucine	3.6	----	3.6	6.0
Leucine	7.2	7.1	7.2	10.0
Lysine	4.2	4.2	4.2	8.0
Methionine	0.5	0.5	0.5	3.3
Phenylalanine	7.0	6.6	6.8	5.4
Threonine	6.8	6.8	6.8	4.3
Valine	6.9	7.0	7.0	7.3
Tryptophan	0.5	0.5	0.5	1.2

[1] The amino acid values are calculated to 16 per cent nitrogen content of the samples.
[2] Total nitrogen of the air-dried material was 9.1 per cent and protein (N x 6.25), 56.9 per cent.

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TABLE 2
Percentages of lysine, methionine, and tryptophan in proteins of bird's nest, meat, fish, and peanut [1]

Foodstuff	Lysine %	Methionine %	Tryptophan %	Reference
Beef muscle	8.0	3.3	1.3	Block and Bolling (*2*)
Pork muscle	8.7	3.4	1.3	Beach et al. (*1*)
Beef liver	6.0	2.4	1.8	Beach et al. (*1*)
Tuna, canned	8.7	2.8	1.2	Murphy and Dunn (7)
Peanut	3.1	1.1	1.0	Murphy and Dunn (7)
Bird's nest	4.2	0.5	0.5	This paper

[1] The amino acid values are calculated to 16% nitrogen content of the samples.

TABLE 3
Cost of bird's nest protein compared to milk, meat, and fish protein[1]

Product	Cost per lb.	Protein in[2] foodstuff %	Cost of protein per lb.
Skimmed milk, dried	$0.33	38	$0.86
Beef, lean	0.98	20	4.90
Pork chops, lean	0.75	17	4.50
Salmon, red	0.83	21	4.70
Bird's nest	5.00	56.9	8.80

[1] Costs calculated on basis of market in Los Angeles as of August, 1956.
[2] Taylor, C. M., and Macleod, G. Rose. *Laboratory Handbook for Dietetics.* 1949, The Macmillan Company, New York.

6 amino acids, and that it has relatively low percentages of 4 amino acids (isoleucine, lysine, methionine, and tryptophan), compared to casein. Compared to other foodstuffs of recognized high nutritional quality, bird's nest protein is deficient in lysine, methionine, and tryptophan (Table 2). The cost of bird's nest protein ranges from 2 to 10 times that of other common foods (Table 3).

SUMMARY

The percentages of the ten essential amino acids in bird's nest have been determined by microbiological assay procedures. It has been concluded that bird's nest protein is not only more costly than other common food proteins, but also inferior nutritionally.

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RETROGRADATION IN A STARCH JELLY CANDY

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The change of rheological properties in a starch gel during aging has been taken as an indication of increasing retrogradation, *i.e.*, crystallization, via the parallelization of linear portions of the starch molecules *(28)*. In order to test this assumption, it is necessary to consider the nature of crystallinity in polymers and the properties of polymeric crystals.

Conceptually, crystallinity is more basically the property of a structure in which there occurs a lattice arrangement of repeating groupings than the property of a structure with a certain external morphology *(32)*. The existence of regular, minute spacings between repeating planes of atomic groupings permits the diffraction of very short wave lengths of electromagnetic radiation, such as X-rays, and thus gives rise to that prime criterion of crystallinity, the X-ray diffraction pattern *(2, 8)*. With the use of this tool, crystalline structure has been found in starch grains, cellulose, wool, stretched rubber, and other high polymeric materials *(22)*.

Inasmuch as the individual polymer molecule is generally quite long, it is believed that a single molecular chain may participate in several, relatively short, crystalline groupings or micelles *(4, 6, 22, 23)*. The regions of the molecular chain not thus organized are considered to be part of the disordered or "amorphous" phase of the compound. Because all sorts of molecular associations may exist, as transitional phases between an ideally random state and a perfectly crystalline organization, there can be no real qualitative distinctions between so-called "crystalline" and "amorphous" phases in a polymeric material. However, quantitative expressions usually have been given for the degree of order (*i.e.*, "crystallinity") which is presumed to obtain in a particular sample.

Various approaches have been undertaken in the estimation of the relative amounts of these two phases *(9, 24)*. Hermans and Weidinger *(7)* evaluated the crystalline proportion of cellulosic products by comparing the intensity of the X-ray interferences with that of the "background," as did also Field *(3)* with rubber. Changes in density have been taken to reflect changes in the relative proportions of the two phases *(29)*. In assaying the retrogradation of starch pastes and bread crumb, Katz *(10)* introduced such indices as the swelling volume, solubility in cold water, and firmness (of crumb). Other workers *(22, 23, 30)* considered that differences in resistance to chemical attack or to enzymatic digestion would correspond to differences in the relative proportions of crystalline and amorphous phases.

These approaches are based upon the consideration that the crystalline areas have extensive intermolecular bonds. Such bonds are supposed to

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render the participating molecular groups less reactive to solvation and to chemical and mechanical disruption. In the case of starch and cellulose, the hydrogen bonds between hydroxyl groups serve this purpose *(18)*.

The present work indicates the results of applying various measures of crystallinity to a starch gel of known rheological properties, at various ages, in order to evaluate the extent of retrogradation with time.

MATERIALS AND METHODS

The starch jellies used in these experiments were of the same composition as described earlier *(27)*. However, specimens for X-ray diffraction study were poured to a constant thickness of 1 mm. by using appropriate spacers between two glass plates. The hot jelly was poured on one glass plate, between the spacers, and the second glass plate was pressed down (on the jelly mass) until it reached the spacers. The assembly was wrapped in "Saran Wrap" and stored.

All the X-ray patterns (for 1, 2, 5, 10, 30, and 60 day old samples) were made the same day, and the photographic films were developed simultaneously. (Pouring of the jelly took place the given number of aging days before the X-ray diffraction experiment.) The Saran covering was removed from each sample, and the glass plates were rotated gently in opposite directions and slipped apart. A rectangular portion of the jelly slab was cut out, mounted on a sample holder, and exposed to Cu-Kα radiation.

It was also believed that optical density would indicate the light-scattering capacity of the starch gel system, reflecting the number and size of crystalline micellar regions. A sample jelly was poured into a rectangular spectrophotometric absorption cell (10 × 10 mm. cross section), into which was then inserted a spacer of 10 × 8 mm. cross section. This arrangement shortened the light path through the jelly to 2 mm. and thereby permitted determination of optical density. These determinations were made at a wave length of 9000 Å (*cf. 31*), at which the initial optical density was at its lowest feasible value. Readings were taken at the same jelly ages as indicated above.

Although it seemed that changes in vapor pressure over the gel would reflect changes in the water-holding capacity of the gel system, hence proportionate crystallinity, this approach was rejected because of theoretical considerations regarding the sensitivity of measurement (*cf.* also the negative results of Platt, *25*). In its place, a measurement of changes in osmotic pressure was attempted. Since diffusion through the gel is quite slow, the gel samples were dispersed in water, to a 10× dilution, by the action of a Waring blender for 5 minutes. A sample of the non-starch components of the gel system, in proportional concentration, was prepared by cooking these in the same manner as in the ordinary gel and dispersing them to a 10× dilution in water to provide the solution about the osmotic cell. Both solutions had added 0.001% phenyl mercuric nitrate for the inhibition of microbial activity. The osmotic cells were Bull osmometers, with Visking cellulose membranes, which were immersed in a 29.0 ± 0.1° C. water bath. However, due to the continual precipitation of starch in the cell during several days of measurement, it was not possible to obtain the osmotic pressure of the original gel solution.

For measuring cold-water solubility of the starch and its resistance to enzymatic attack, a common preparative scheme was used. About 30 g. of gel samples were weighed and then blended with a weighed amount of distilled water, buffered to pH 5.6, for 5 minutes. While the slurry was being stirred, 25 ml. aliquots were removed to tared moisture dishes, weighed, dried in a vacuum oven (26 inches Hg, 70° C.) for 48 hours, and weighed again for a determination of total solids. The amount of water-soluble solids was measured as follows: weighed 25 ml. aliquots of the original slurry were centrifuged at 4400 *g* for 5 minutes, the decantate saved, the centrifugate mixed with fresh buffer solution and centrifuged for another 5 minutes, and the second decantate combined with the first and weighed. Aliquots from this "combined decantate" were weighed in tared moisture dishes, dried in a vacuum oven (as specified above), and weighed again. This procedure permitted a calculation of the quantity of cold water-soluble material (including sugars and ash). The difference between this value and the total solids content was the amount of water-insoluble solids.

To obtain the cold water-soluble starch, aliquots of the combined decantate were treated with hot 95% ethanol, giving a final concentration of 80% alcohol, in tared centrifuge tubes. Centrifuging, pouring off the decantate, further mixing with hot 95% ethanol, and again centrifuging and pouring off the decantate gave a solid residue, which was assumed to be true "starch." The weight of the vacuum-dried residue, referred to the weight of the aliquot before treatment with alcohol, was considered to give a measure of cold-water soluble starch.

A further estimate of the cold-water soluble starch was given by an iodine sorption technique. An aliquot of the same combined decantate (above) was diluted (1:20) with dilute Lugol's solution (2 gm. KI, 1 g. I_2, 97 g. H_2O, diluted 1:100 with pH 5.6 buffer solution) and its optical density determined at 5900 Å, with a 0.1 mm. slit. Readings of optical density were also taken on a buffer solution blank, the diluted Lugol's solution, and another diluted (1:20) aliquot of the combined decantate. The last two solutions gave controls on the absorption of light by the iodine and on the turbidity and sugar absorption background, respectively.

Enzymatic digestibility was determined by taking 25 ml. of the original jelly slurry during stirring, weighing it, and adding 75 ml. of β-amylase solution (0.05 g. in 75 ml. of pH 5.6 buffer solution). The mixture was stirred for 6 hours in a $29.0 \pm 0.1°$ C. water bath. Following digestion, aliquots were weighed in tared centrifuge tubes. These were then extracted with hot 95% ethanol (to a final concentration of 80% alcohol) and centrifuged. The process was repeated, after decantation, with added hot 95% ethanol, and the decantate again poured off. The centrifuge tubes were dried in a vacuum oven and weighed, giving the amount of undigested material. (Control solutions of enzyme were also treated in this manner.)

Gel density was ascertained by weighing the gel, in a small wire basket, in air and in toluene. From each value was subtracted, respectively, the weight of the basket in air and in toluene. Gel density was calculated as follows:

$$\text{gel density} = \frac{(\text{weight of gel in air}) \times (\text{density of toluene})}{(\text{weight of gel in air}) - (\text{weight of gel in toluene})}$$

The maximum temperature variation between measurements was 3° C.; this was corrected according to values given in the International Critical Tables for toluene density.

RESULTS AND DISCUSSION

Virtually all the presumptive measures of crystallinity show a progressive change with time. Figures 1, 2, and 3 portray, via positive photographic prints of the X-ray diffraction patterns, the increasing number and increasing sharpness and intensity of the rings of various lattice spac-

Figure 1. X-ray diffraction pattern of 1-day gel. **Figure 2.** X-ray diffraction pattern of 5-day gel. **Figure 3.** X-ray diffraction pattern of 30-day gel.

All figures are positive prints from film exposed at 5 cm. from sample with Cu-Kα radiation. ($\times$ 0.77)

TABLE 1

Variation of starch jelly attributes with age

Age (days)	Cold water-soluble solids		Cold water-insoluble solids (%)[1]	Unhydrolyzable solids (%)[1]	Optical density of gel	Density g./cc.
	Alcohol precipitate (%)[1]	Optical density of iodine sorption				
1	7.43	0.657	2.30	6.02	0.071	1.388
2	5.10	0.591	2.93	3.73	0.088	1.387
5	4.25	0.494	3.59	3.44	0.118	1.394
10	3.35	0.221	5.80	4.24	0.133	1.395
30	2.36	0.220	7.66	4.62	0.137	1.398
60	1.84	0.132	8.19	6.52	0.167	1.402

[1] On fresh jelly weight basis.

ings for the 1, 5, and 30 day old gels, respectively. Note also the decreasing intensity of the amorphous background in these figures. Likewise, Table 1 shows the decreasing amount of cold water-soluble starch (measured both gravimetrically and iodometrically), the corresponding increase of cold water-insoluble solids, and the increasing opacity of the gel as functions of gel age. Except for the 1-day and 2-day determinations of enzymic digestibility (which may be due to experimental error or possible microbial contamination), that measurement also shows a change in the direction of decreasing availability to enzymatic attack. [Although Schoch and Elder *(26)* point out the presumptive strongly crystalline, hence difficultly reactive, structure of wrinkled pea starch, Maige *(21)* finds that this starch is digested fairly readily during seed germination. Wrinkled pea starch is composed of a relatively large proportion of amylose, as is presumably also the acid-modified corn starch used here *(18).*] Changes in gel density are small, following the trend in moisture content *(28);* however, it is not warranted to accept these small changes as reflecting an increasing degree of crystallinity.

When the data are construed graphically, with a logarithmic abscissa for time of aging, it may be seen that the opacity of the gel and the adsorption of iodine in the cold-water soluble material are related rectilinearly (but with different signs) with gel age (Figure 4). It will be recalled *(28)* that the elastic modulus of this gel shows a similar relationship. Perhaps it could be inferred that the elastic modulus depends directly on the relative amount of micellar groupings (which act as points of light scattering). Also, the iodine adsorption would indicate the quantity of starch in micelles by showing what fraction of the starch is *not* so organized.

On the other hand, the logarithms of the amount of cold water-insoluble solids and of the alcohol precipitate of the cold water-soluble material are rectilinear functions of the logarithm of gel age (Figure 5). Therefore, perhaps it may be speculated that the "viscosity" coefficient (the logarithm of which has a rectilinear relationship with the logarithm of time, *28)* depends on the relative proportion of starch molecules which are bound together in the gel network, as compared with those which are not.

Thus, there is indirect, as well as direct, evidence of an increasing percentage of crystalline material with time in the starch gel. Katz *(10, 11, 12)* and other workers *(5)* have shown that the crystalline nature of a

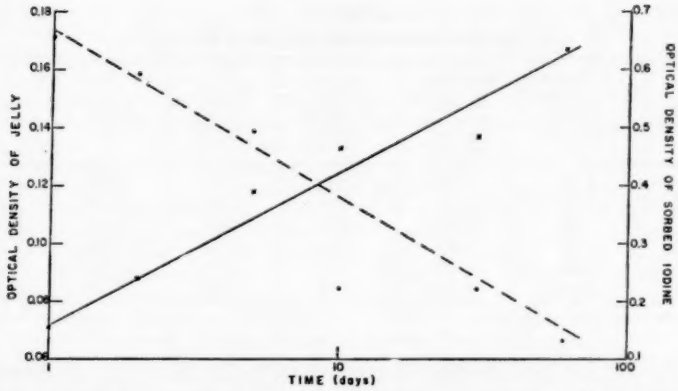

Figure 4. Optical density of jelly (solid line, crosses) and optical density of sorbed iodine in cold water-soluble fraction (broken line, circles) as functions of the logarithm of gel age.

starch paste changes during the period following its heating and consequent gelatinization. Specifically, the so-called V-pattern of gelatinized starch (which is characterized by the 7.17 Å, 4.45 Å, and 2.79 Å spacings, *11)* is said to be gradually superimposed by a B-pattern (characterized by spacings of 15.9 Å, 4.04 Å, and 3.69 Å), which almost, but not completely, displaces the former *(10, 11, 12)*. A consideration of the changes in X-ray pattern of the corn starch confectionery gel (Figures 1-3 and Table 2) permits a different explanation to be made for the crystallization changes in that gel. The significant changes here appear to be in the direction of delineation of new rings, to be sure, but also in the intensification of the

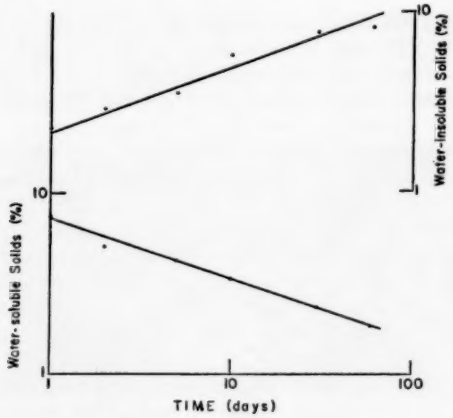

Figure 5. Logarithms of per cent of alcohol precipitate of cold water-soluble fraction of gel (below) and of per cent of cold water-insoluble fraction (above) as functions of the logarithm of gel age.

TABLE 2

X-ray data of corn starch jelly, compared with those of other cereal starches

Corn starch jelly		A[1]		V[1]		B[1]	
d[2]	I[3]	d	I	d	I	d	I
				2.79	m		
3.77	vf	3.81	s			3.61	m
4.12	f					3.98	m
4.65	m	4.45	f	4.45	s	4.40	f
5.37	s	5.05	s			5.10	s
		5.98	m			5.84	m
6.12	vf					6.13	m
7.04	f			7.17	m		
						8.34	f
16.1	s					15.9	s

[1] A is pattern of native cereal starch, V of gelatinized starch, and B of retrograded starch *(11, 17)*.
[2] Interplanar spacings, in A, corresponding to diffraction interferences observed.
[3] Intensity of interferences on photographic plate: s, strong; m, medium; f, faint; vf, very faint.

first-appearing rings during the course of aging. Note, for example, that the innermost sharp ring for the 7.04 Å reflection becomes more intense rather than fainter. Similar X-ray changes may be found in gelatin gels *(15, 16)*, in which there occurs likewise an increase in strength and in elastic modulus from sol to gel state and during gel aging *(19, 20)*. It is, of course, possible to explain the series of spacings in Table 2 as representing a crystalline configuration in which both starch and sugar participate. Nevertheless, the relationship between age and increasing crystallinity in the gel is apparent.

Although the breadth of the diffraction rings (an indication of the presence of very tiny crystalline regions) precludes extremely accurate measurement of the ring diameters, measurements to the nearest 0.1 mm. show no significant changes in diameter with age in the rings at the 4.12 Å, 4.65 Å, and 5.37 Å positions. Hence, the corresponding interplanar distances in the crystal lattice scarcely change at all during aging. Since water is sequestered during retrogradation *(11)*, this could indicate that, as in crystalline cellulose I, the molecules of water do not participate in the formation of this particular polysaccharide (starch) crystal in the gel. [Note the contrasting results for inulin *(14)* and pectic substances *(1)*.]

Because of the relatively low intensity of the rings, in comparison with the background, photometric measurements are inadequate to reveal differences in relative intensity as a function of age. However, Katz *(13)* has commented on the ability of the human eye to perceive differences in light intensity, which cannot be recorded instrumentally. Hence, it is to be noted that a visual comparison of Figures 1, 2, and 3 will indicate the continual increase in pattern intensity, over the background, with age.

A note should be added concerning unmeasured changes which occur during aging of the gel. One of these is the increasing wetness of the gel surface. The "starchy" adhesiveness of the freshly-poured gel succeeds to a wetter stickiness; *i.e.*, "sweating" occurs. Also, successively older gels are more difficult to weigh accurately because of their more rapid weight loss during their exposure on the balance. These two data are readily interpretable in the light of the evidence presented above: crystallization,

via hydrogen bonding between starch molecules, occurs at the expense of starch-water bonding, and thus there is an active elimination of water from the gel network (recall that water is not a part of the polysaccharide crystalline unit here). If the vapor pressure is too high (as when the gel is enclosed by Saran Wrap), the water vapor condenses in the syrup fraction about the polymer reticulum and on the jelly surface, leading to stickiness. When the Saran film is removed, the vapor pressure decreases, and the now lightly-held water can escape readily by evaporation.

SUMMARY

Retrogradation (*i.e.*, crystallization) was measured in aging starch gels by various methods (including cold water-solubility, X-ray diffraction, opacity, iodine sorption, and enzymatic digestion). Most methods clearly showed an increase of crystallinity with time, but the rate of increase differed among them. Aging brought about no changes in the lattice dimension of the polysaccharide crystal. Certain other properties of the older gel were discussed.

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EVALUATION OF FLAVOR CHANGES DUE TO PESTICIDES—A REGIONAL APPROACH[a]

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Flavor changes in canned and frozen fruits and vegetables caused by pesticides are influenced not only by the type and concentration of pesticide used but also by the time and method of application of the pesticide and the kind of crop, whether root or above ground. Additional factors which may modify or intensify pesticide effects on flavor are climatic or environmental conditions, pre-processing and processing techniques, and the delayed effects of accumulated pesticide residues. These changes have recently become a serious problem to the food processing industry because oftentimes flavor is adversely affected.

To cope with this situation a group of Northeast agricultural experiment stations, namely, Connecticut, Maine, Maryland, Massachusetts, New York (Cornell) and Pennsylvania initiated a regional project to study methods of measuring flavor changes in processed fruits and vegetables subjected to pesticidal treatments in carefully designed field plots.

The objectives of this two year investigation by the six Northeast experiment stations were:

a. To study under regional conditions the adaptability of a multiple comparison or variables method for measuring pesticidal flavor changes.
b. To develop a standard or uniform procedure to serve as a guide for other work in evaluating flavor changes, thus helping to standardize reporting and evaluating of experimental results.

EXPERIMENTAL

Materials and Methods. The materials and methods of preparation are shown in Table 1. Samples were stored at room temperature and tested simultaneously at all locations.

Methods of scoring and analysis of data. Figure 1 shows the scoring form used by the judges in 1954-5. All treatments of the product being evaluated were tested by the judges at one sitting. Order of presentation was varied at random for each of 4 replications.

In 1955-6, a sample of the check treatment labeled as "standard" was provided and the judges instructed to use this as a basis for comparison with the flavor of the

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[b] Maryland, [c] New York (Cornell), [d] Massachusetts, [e] Pennsylvania, [f] Maine.

[g] Presented at the Sixteenth Annual Meeting of the Institute of Food Technologists, St. Louis, Missouri, June 13, 1956.

TABLE 1

Products used for panel evaluation

Season	Product	Pesticidal Treatments	Grown	Prepared		Panel	
				Location	Method	Location	Size[b]
1954-5	Squash	3 + check	Mass.	Mass.	Canned Puree	Maine	44-8
						Maryland	19-4
						Mass.	28-5
	Apple	3 + check	Conn.	Mass.	Canned Sauce	Maine	45-7
						Maryland	17-4
						Mass.	21-4
1955-6	Squash	3 + check	Mass.	Mass.	Canned Puree	Maine	29-9
						Maryland	--6
						Mass.	25-7
						Cornell	--5
						Pa.	25-6
	Apple	4 + check	Conn.	Mass.	Canned Sauce	Maine	--8
						Maryland	--7
						Mass.	18-7
						Cornell	--12
						Pa.	18-6

[b] First value in the hyphenated number indicates number of judges in the original large untested group; second value is the final number of judges in the selected panel.

unknown samples which were rated by checking one of the categories listed on the score card (Figure 2). Numerical values which are in parentheses at the bottom of Figure 2 were later assigned to each category for statistical treatment of the data.

There were four major changes in the score card from the 1954-5 form (Figure 1).

1. Samples were rated for flavor in comparison to an actual standard sample rather than against a hypothetical average flavor.

2. All numbers were eliminated, the judge merely placed a check in a descriptive block or cell.

3. An additional point "Below standard in flavor—no detectable off-flavor" was added thereby expanding the scale to five points.

4. The additional column "Describe off-flavor if possible" and a list of suggested words was added.

All data were analyzed by the standard analysis of variance procedure (*3*).

Panel screening. The judges were screened at each location for each commodity in an attempt to acquire a trained panel. Thus:

a. A large untested panel scored each treatment of each product 4 times. The judges' individual scores were then analyzed by the analysis of variance and those judges showing statistical significance at odds of 9:1 for treatments were retained for further testing.

b. The retained judges retested the product and were dropped from the panel if their F values for treatments were not significant at odds of 19:1.

c. The final panel of selected judges retested the product 4 times and their data were used to determine the magnitude of differences among treatments.

In 1955-6 some stations did not screen the panel because they already had available a trained panel from earlier work or used the analyses from the range (*2*), quick method.

Measurement of panel efficiency *(1)*. Panel efficiency was measured in terms of number of individual tastings required for demonstrating statistical significance at the 1% level. This was accomplished by the use of "sample number ratio" (SNR) multiplied by the number of tastings used (N). SNR may be obtained by the following equation:

$$\text{SNR} = \left(\frac{\text{LSD 1\% for treatment means}}{\text{Range for treatments}} \right)^2$$

FLAVOR EVALUATION

RATE FLAVOR ONLY—IGNORE ALL OTHER DIFFERENCES

Date: _____ Project NE15 ( )

Name of Judge: _____ Product: _____

Code No.: _____

Sample No.	Flavor Only	Remarks
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

RATING SCALE

4—Better than average in flavor

3—Average flavor

2—Slightly off flavor

1—Definitely off flavor not acceptable

INSTRUCTIONS FOR JUDGE

1. Using the rating scale, enter by the sample number the rating number you think best describes the flavor of the sample.
2. Please, no fractional scoring of samples!

Fig. 1. Flavor evaluation sheet—1954-5.

The LSD 1% is the calculated least significant difference at the 1% level (*3*). Range is determined by subtracting the smallest mean treatment value from the highest. The number of tastings required for demonstrating significance may then be obtained by multiplying the SNR by N, where N=number of tastings actually used in the test.

Least practical difference *(1)*. The term "least practical difference" (LPD) was coined to indicate differences of practical magnitude, in contrast to the LSD term which indicates differences that are statistically significant, but may not necessarily be of practical importance. Thus for example, if an investigator decides that a unit difference on the flavor scale is of practical magnitude, the LPD would be 1.0. The use of the following formula will give a suitable basis for setting the number of tastings required for obtaining practical significance at this level.

$$\text{N required} = \left(\frac{\text{LSD 1\% for treatments}}{\text{LPD}}\right)^2 \times \text{N used}$$

Thus, if LSD 1% is 0.22, and the LPD is set at 1.0, and 600 tastings were used, then the number of tastings required to show a significant difference of practical magnitude would be:

$$\text{N required} = \left(\frac{.22}{1.00}\right)^2 \times 600, \text{ or } 29$$

FLAVOR EVALUATION

RATE FLAVOR ONLY—IGNORE ALL OTHER DIFFERENCES

Date _____ Project code _____

Name _____ Product _____

CHECK ONE:

Sample	Better than standard in flavor	Equal to standard in flavor	Below standard in flavor No detectable off-flavor	Slightly Off Flavor	Definitely Off Flavor Not Acceptable	Describe Off Flavor[1] if possible
A						
B						
C						
D						
E						
F						
	(5)	(4)	(3)	(2)	(1)	

[1] List of suggested words:

Bitter	Flat	Metallic	Putrid	Salty	Sweet
Burnt	Fragrant	Musty	Puckery	Sour	Woody
Earthy	Medicinal	Oily	Rancid	Spicy	

Figure 2. Flavor evaluation sheet—1955-6.

RESULTS AND DISCUSSION

1954-5

Squash. Squash which was tested by the large untested panels at Laboratories 1, 2, and 3 (hereafter designated Lab 1, Lab 2, Lab 3, etc.) showed a significant flavor difference among treatments and check (Table 2A). Lab 1 found samples 35 and 36 significantly lower than the check in flavor. Lab 1 was much more efficient than the other two and required only 56 tastings to obtain statistical significance at the 1% level. Lab 2 rated second in panel "power."

In Table 2B are shown the results of the second panel's evaluation. Panel size was reduced to 43, 42, and 39% of original size. F values for treatments were significant at the 1% level. At Lab 1 there was a highly significant *treatment* and *judge* (T×J) interaction. The ranking of the samples was not identical but sample 35 was rated significantly lower in flavor than the check at all stations. Lab 1 rated highest, Lab 3 second, and Lab 2 last in panel power.

TABLE 2

Comparison of panel performances from three laboratories for squash 1954-5

	Treatment	Lab 1 - 44 judges		Lab 2 - 19 judges		Lab 3 - 28 judges	
		Score	Rank	Score	Rank	Score	Rank
A. Untested Panel	34	2.72	1	2.72	3	2.64	1
	35	1.93	4	2.24	4	2.09	4
	36	2.18	3	2.74	2	2.36	3
First Screening	Check	2.60	2	2.76	1	2.48	2
	LSD 1%	.23		.31		.36	
	Range	.79		.52		.55	
	SNR	.08		.36		.42	
	No. of tastings						
	used	704		304		448	
	required	56		109		188	
B. Second Screening			*19 judges*		*8 judges*		*11 judges*
	34	2.53	1	2.63	2	2.45	2
	35	1.34	4	2.16	4	1.93	4
	36	1.96	3	2.41	3	2.34	3
	Check	2.34	2	2.75	1	2.73	1
	LSD 1%	.45		.57		.58	
	Range	1.19		.59		.80	
	SNR	.14		.94		.53	
	No. of tastings						
	used	304		128		176	
	required	43		120		93	
	% of original						
	panel	43		42		39	
C. Selected Panel			*8 judges*		*4 judges*		*5 judges*
	34	2.80	1	2.63	2	2.65	2
	35	1.36	4	1.93	4	1.75	4
	36	1.97	3	2.31	3	2.40	3
	Check	2.66	2	2.75	1	2.75	1
	LSD 1%	.38		.76		1.76	
	Range	1.44		.82		1.00	
	SNR	.07		.86		3.09	
	No. of tastings						
	used	128		48		80	
	required	9		41		247	
	% of original						
	panel	18		21		17	

A selected panel retested the samples, with a panel size reduction to 18, 21, and 17% of the original panel. Lab 1 again showed a significant T×J interaction. The panels still were not in exact agreement, with Lab 2 and Lab 3 rating samples in an identical manner but with Lab 1 reversing the positions of samples 34 and check. All panels were in agreement in rating sample 35 significantly lower than check in flavor. Lab 1 also judged sample 36 to be lower than the check in flavor. Again Lab 1 showed highest panel efficiency with Lab 2 next and Lab 3 showing an actual increase (93-247) in number of tastings required for significance at the 1% level.

TABLE 3

Comparison of panel data from three laboratories showing the combined results of selected panels[1]

Treatments	Squash 1954-5							
	Lab 1		Lab 2		Lab 3		Combined	
	Score	Rank	Score	Rank	Score	Rank	Score	Rank
34	2.40	2	2.75	2	2.65	2	2.60	2
35	1.00	4	2.00	4	1.75	4	1.58	4
36	1.70	3	2.45	3	2.40	3	2.18	3
Check	2.45	1	2.81	1	2.75	1	2.67	1
					LSD 1%		.44	
					Range		1.09	
					SNR		.40	

F Table

Variance Source	F
Treatment	20.13**
Judge	1.58
Location	11.48**
T × J	1.01
T × L	0.78
J × L	2.47*
Tastings used	240
required for:	
LPD[2] 0.5	185

[1] Four top ranked judges only.
[2] Least "practical" difference.
* Significant at the 5% level.
** Significant at the 1% level.

In Table 3 the combined scores using only the selected judges showed a significant flavor difference between treatments. Samples 35 and 36 were rated significantly lower than check in flavor when data from the 3 experiment stations were combined in this manner. The location X judge interaction and location significance indicated a different reaction of the judges at the various locations. If a Least "Practical" Difference of 0.5 was desired instead of the actual LSD 1% level of .44, only 185 tastings would have been required instead of the 240 tastings which were used (5 judges × 4 treatments × 4 replications × 3 locations). This indicates for example that with these laboratories, judges and samples the desired LPD could have been achieved by 12 selected judges × 4 treatments × 2 replications at 2 locations.

Apple sauce. In the original large untested panels shown in Table 4A the treatment F values were not significant. The ranking of the samples varied considerably and the average range was about .17 flavor scale units. Lab 1 was rated first in efficiency with Lab 2 second in efficiency. Lab 3 required a tremendous number of tastings to evince treatment significance.

The evaluation by the second group, Table 4B, again showed no significance between treatments. The panel size was reduced to 15, 48, and 23% of the original. In the second screening all groups needed a great many more tastings to acquire F value significance for treatments.

TABLE 4

Comparison of panel performances from three laboratories for apple sauce 1954-5

	Treatment	Lab 1 - 45 judges		Lab 2 - 17 judges		Lab 3 - 21 judges	
		Score	Rank	Score	Rank	Score	Rank
A. Untested Panel	R	2.70	1	2.79	1	2.62	4
	RW	2.60	2.5	2.69	4	2.83	1
	W	2.60	2.5	2.72	3	2.63	3
First Screening	G-Check	2.50	4	2.78	2	2.70	2
	LSD 1%	.20		.78		2.36	
	Range	.20		.09		.21	
	SNR	1.00		74.99		1.25	
	No of tastings						
	used	720		272		336	
	required	720		20,397		420	
B. Second Screening			*7 judges*		*10 judges*		*4 judges*
	R	2.61	4	2.83	2	2.87	1.5
	RW	2.71	2	2.78	4	2.37	4
	W	2.68	3	2.80	3	2.50	3
	G-Check	2.75	1	2.95	1	2.87	1.5
	LSD 1%	.59		.94		1.66	
	Range	.14		.17		.50	
	SNR	17.72		30.47		11.02	
	No. of tastings						
	used	112		160		64	
	required	1985		4875		705	
	% of original						
	panel	15		48		23	
C. Selected Panel					*4 judges*		
	R			2.75	3		
	RW			2.94	2		
	W			2.69	4		
	G-Check			3.06	1		
	LSD 1%			2.19			
	Range			.37			
	SNR			34.92			
	No. of tastings						
	used			64			
	required			2235			
	% of original						
	panel			11			

The Lab 3 group used a selected group of judges eliminating those from the first screening whose F values for treatment were not significant at the 5% point. This reduced the panel to 11% of the original group.

This selected group found no significance between treatments. The tastings required to get significant F values for treatment were 2233 compared with 64 actual tastings.

In combining data (Table 5) of 4 selected judges from each of the three locations, using data from the last test only, there was still no significance between treatments but highly significant F values for judges,

TABLE 5

Comparison of panel data from three laboratories showing the combined results of selected panels[1]

Treatment	Apple Sauce 1954-5							
	Lab 1		Lab 2		Lab 3		Combined	
	Score	Rank	Score	Rank	Score	Rank	Score	Rank
R	2.63	2	2.87	1.5	2.75	3	2.75	3
RW	3.00	4	2.37	4	2.94	2	2.77	2
W	2.63	2	2.50	3	2.69	4	2.60	4
G-Check	2.63	2	2.87	1.5	3.06	1	2.85	1
					LSD 1%		.59	
					Range		.25	
					SNR		5.57	

F Table

Variance Source	F
Treatment	2.06
Judge	9.13**
Location	2.80
T × J	1.41
T × L	4.82**
J × L	5.37**
Tastings used	182
required for: LSD 1%	1014

[1] Four top ranked judges only.
** Significant at the 1% level.

T×L and J×L interaction which indicated treatments were evaluated differently at different locations, and judges reacted differently at different locations.

1955-6

Squash. In the squash study the development of trained panels was compared at 3 stations. The other 2 stations used a slightly different method or used the selected panel from 1954-5. All stations used a 5-point rating scale and a marked standard reference sample.

At Labs 1, 3 and 5 the large untested groups (25-29 judges) were able to distinguish between treatments at the 1% level, (Table 6A). At Lab 1 the T×J interaction was significant at the 1% level. The addition of another point to the scale and the use of a reference standard sample seemed to have had little effect on reducing T×J interaction significance. The large untested panels were entirely in agreement in ranking the samples. In general the X sample ranked higher in flavor than any of the others, and was significantly better than check in flavor. Lab 3 rated highest in panel efficiency with Labs 1 and 5 being very close. In general it appeared that only about one-fourth of the actual work was needed to get the desired level of significance.

In the second screening, panel sizes were reduced to 34, 44 and 48% of the original panel. This average reduction to 42% compares favorably

TABLE 6

Comparison of panel performances from three laboratories for squash 1955-6

	Treatment	Lab 1 - 29 judges		Lab 3 - 25 judges		Lab 5 - 25 judges	
		Score	Rank	Score	Rank	Score	Rank
A. Untested Panel	Check	3.41	3	3.29	3	3.64	3
	X	4.19	1	3.79	1	4.35	1
	Y	4.17	2	3.77	2	3.80	2
First Screening	Z	3.29	4	3.10	4	3.23	4
	LSD 1%	.46		.21		.57	
	Range	.90		.69		1.12	
	SNR	.26		.09		.25	
	No. of tastings						
	used	464		400		400	
	required	121		36		100	
B. Second Screening			*9 judges*		*11 judges*		*12 judges*
	Check	3.58	3.5	3.52	3	3.71	3
	X	4.50	1	3.89	2	4.00	1
	Y	4.17	2	4.13	1	3.85	2
	Z	3.53	3.5	3.05	4	3.25	4
	LSD 1%	.70		.68		.66	
	Range	.97		1.08		.75	
	SNR	.52		.40		.77	
	No. of tastings						
	used	144		176		192	
	required	75		70		148	
	% of original						
	panel	34		44		48	
C. Selected Panel					*7 judges*		*6 judges*
	Check			3.39	4	3.88	3
	X			4.28	1	4.92	1
	Y			4.14	2	4.12	2
	Z			3.54	3	3.38	4
	LSD 1%			.41		.88	
	Range			.39		1.54	
	SNR			1.1		.32	
	No. of tastings						
	used			112		96	
	required			123		31	
	% of original						
	panel			28		24	

with a reduction to 41% in panel size at the same point in the 1954-5 work.

Second screening results of judges at Lab 1 and Lab 5 showed a significant T×J interaction. As shown in Table 6B the different locations ranked X sample generally higher in flavor and Z sample lower in flavor than the check. The ranking was very similar to that of the large untested panel. Lab 3 was rated as most efficient, with Lab 1 second and Lab 5 last.

At the end of the second screening Lab 3 and Lab 5 reduced the panel size to 28 and 24% of the original. This 26% reduction of the original panel size may be compared with a reduction to 17% in 1954-5 when no standard reference sample was used.

In the final results of the selected panels (Table 6C) Lab 3 gave somewhat different results from Lab 5. Lab 5 data showed highly significant treatment, and T×J interaction while Lab 3 showed no significance for treatments or judges. The latter situation was an exact repeat of the 1954-5 work in which the selected panel showed no significance between treatments although they had done so throughout the entire training period. This pointed out the danger of relying entirely on a small selected group of tasters where poor performance on one day would skew results. Lab 5 ranked highest with the Lab 3 data showing a need for 123 tastings compared to the actual 112 to get treatment significance at the 1% point.

The 5-point scale increased the treatment range from 1.09 in 1954-5 to 1.29 for 1955-6 with about the same treatments. Sample Z was significantly lower than in flavor at the 1% level and sample Y rated significantly higher than check. The 2.8 mean value for sample Z probably indicated some definite flavor change attributable to treatment. This flavor was described as both bitter and sour by the judges. Such results indicated the need for further study of the effect of this treatment.

TABLE 7
Comparison of panel data from five laboratories showing the combined results of selected panels[1]

Treatment	Squash 1955-6							
	Lab 1		Lab 2		Lab 3		Lab 4	
	Score	Rank	Score	Rank	Score	Rank	Score	Rank
Check	3.28	3	4.02	1	3.30	3	4.08	1
X	4.64	1	3.58	3	3.68	2	4.36	2
Y	4.46	2	3.76	2	4.16	1	3.74	3
Z	2.88	4	2.52	4	2.76	4	2.01	4

Treatment	Lab 5		Combined	
	Score	Rank	Score	Rank
Check	4.02	3	3.75	3
X	4.92	1	4.04	2
Y	4.42	2	4.10	1
Z	3.78	4	2.81	4
LSD 1%			.35	
Range			1.29	
SNR			.07	

F Table

Variance Source	F
Treatment	41.34**
Judge	7.39
Location	13.86**
T × J	1.53
T × L	.42
J × L	2.18*
Tastings:	
used	400
required for:	
LPD 0.5	196

[1] Five top ranked judges only.
* Significant at the 5% level.
** Significant at the 1% level.

Four hundred tastings were involved in Table 7. If an LPD of 0.5 is desired as compared with the actual LSD 1% level of .35 only 196 tastings would be needed. This need would be satisfied by 9 trained judges × 4 treatments × 2 replications × 3 locations which is about ½ the work actually done.

The location factor was again highly significant even with the use of a the same labeled standard at each location. The T×J interaction was not significant which agreed with the 1954-5 work.

Apple sauce. Lab 3 and Lab 5 carried out a complete screening of judges while Lab 1, Lab 2, and Lab 4 either used a slightly different screening system or used selected judges.

TABLE 8

Comparison of panel performances from two laboratories for apple sauce 1955-6

	Treatment	Lab 3 - 18 judges		Lab 5 - 18 judges	
		Score	Rank	Score	Rank
A. Untested Panel First Screening	Check	3.72	1	3.76	2
	R	3.58	2	4.21	1
	B21	3.22	5	3.68	3
	B23	3.55	3	3.62	4
	W	3.48	4	2.07	5
	LSD 1%	.44		.64	
	Range	.50		2.14	
	SNR	.77		.10	
	No. of tastings used	360		360	
	required	277		36	
B. Second Screening			*7 judges*		*6 judges*
	Check	4.15	2	4.08	3
	R	4.16	1	4.83	1
	B21	3.43	3	3.96	4
	B23	3.29	4	4.13	2
	W	3.07	5	3.21	5
	LSD 1%	.79		.95	
	Range	1.09		1.60	
	SNR	.53		.35	
	No. of tastings used	140		120	
	required	74		42	
	% of original panel	65		67	

Results of the untested panel at Lab 5 (Table 8A) showed the F value for T×J interaction to be highly significant. Lab 5 rated treatment W as significantly different from check at the 1% point. Lab 3 rated B21 significantly lower than check in flavor. The ranking of the samples was different between laboratories. Lab 5 rated much higher than Lab 3 in panel efficiency.

In the evaluation shown in Table 8B the treatment differences were highly significant at both locations. At Lab 5, sample W was rated be-

TABLE 9

Comparison of taste test data from five laboratories showing the combined results of selected panels[1]

Treatment	Apple Sauce 1955-6							
	Lab 1		Lab 2		Lab 3		Lab 4	
	Score	Rank	Score	Rank	Score	Rank	Score	Rank
Check	4.15	3	4.03	2	4.18	1	3.93	1
R	4.48	1	4.07	1	3.80	2	3.27	2
B21	4.27	2	4.02	3	3.18	4	2.67	4
B23	4.10	4	3.62	4	3.27	3	2.90	3
W	3.10	5	3.37	5	3.02	5	2.57	5

Treatment	Lab 5		Combined	
	Score	Rank	Score	Rank
Check	4.10	2.5	4.08	1.5
R	4.83	1	4.08	1.5
B21	3.98	4	3.62	3
B23	4.10	2.5	3.59	4
W	3.22	5	3.05	5
	LSD 1%		.36	
	Range		1.03	
	SNR		.14	

F Table

Variance Source	F
Treatment	17.79**
Judge	.99
Location	16.53
T × J	1.22
T × L	1.68
J × L	2.81**
Tastings	
used	600
required for:	
LPD 0.5	348

[1] Five top ranked judges only.
** Significant at the 1% level.

low sample R in flavor at the 1% level. At Lab 3, W rated lower than the check at the 1% point. Again Lab 5 rated higher than Lab 3 in panel efficiency.

In Table 9 is presented the summary of selected panels from each of the 5 laboratories for apple sauce tested in 1955-6. Treatment, location, and location × judge interaction were all significant at the 1% level. The T X J interaction was not significant. This is comparable with Table 5 (apple sauce 1954-5) in which judge, treatment × location and location × judge interaction were also significant. All treatments except R were rated significantly lower than check in flavor while treatment W was significantly lower than B21 and B23. A scale point difference of 1 point between check and W might be considered large enough to suspect chemical W was causing some flavor change in the processed apples. Sample W was described as flat and sour by the judges.

SUMMARY AND CONCLUSIONS

In a regional study of methods of evaluating flavor changes in processed fruits and vegetables due to pesticide treatments, three laboratories cooperated one year and five the following year in testing samples of squash and apple sauce.

The multiple comparison or variables method of evaluating samples gave results of reasonable uniformity among the laboratories. Although scores of flavor difference among samples varied somewhat with the laboratories, essentially the same treatments were shown as being significantly different from the checks in flavor when the actual differences were 0.5 scale points or more. Conversely where differences among treatments were less than 0.5 scale points no statistically significant differences were indicated by any of the laboratories.

The use of a balanced factorial design at each station greatly facilitated the combining of data for analysis of variance.

There were certain differences between the way a large untested group of judges (25-45) and a small selected group of judges (4-8) scored canned squash and apple sauce. The large untested groups generally rated lower in panel efficiency where practical differences among samples were demonstrated. On the other hand where treatment differences were not significant the selected panel showed less likelihood of showing erroneous conclusions. The smaller selected panels appeared to give a greater range of differences between treatments when treatments were significantly different.

Since large untested panels are extremely cumbersome and require large amounts of food samples and too small selected panels are affected seriously by individual judges' aberrations it is recommended that panels of from 8-12 selected individuals and 4 replications be used for testing.

The five-point scale used in 1955-6 gave the tasters greater ease in rating the samples than the 4-point scale used in 1954-5.

Descriptive words to describe off-flavors were of value in interpreting results at the various laboratories. However, the use of too many different words to describe a particularly strong off-flavored sample may sometimes cause confusion in evaluating results.

Screening judges on the basis of individual F value for treatments or by the quick method of range ratio (*2*) showed rather uniform reduction in panel size among the laboratories.

No conclusions could be reached as to whether or not there was an increased panel precision and reduction of location significance when a marked standard sample was presented to judges at each laboratory.

The use of the Sample Number Ratio (SNR) $\times$ N to compare panel efficiency

$$\left(\frac{\text{LSD 1\% treatment means}}{\text{Range for treatments}}\right)^2 \times \text{N}$$

gives an estimate of the individual panel's power in terms of tastings needed to get treatment significance at the 1% level.

$$\text{The use of N required} = \left(\frac{\text{LSD 1\% for treatments}}{\text{LPD}}\right)^2 \times \text{N used,}$$

gives a suitable basis for estimating number of tastings required to show a significant difference of practical magnitude (LPD).

The following is a standard or uniform procedure suggested for groups wishing to evaluate flavor changes due to pesticides.

I. Panel of unknown efficiency or wholly untrained persons
   1. Evaluate the test samples with the largest number of people available. Present all treatments (up to 10) in a random fashion replicating 4 times. This should be at 4 different sittings.
   2. Use evaluation form similar to Figure 2, assigning numbers after judging, for statistical analysis.
   3. Use analysis of variance to test for significance of difference.
   4. If treatment F values are not significant and SNR × N values are high there is little likelihood of any real difference between samples. Testing can be discontinued.
   5. If treatment F values are significant further testing is probably required. Screen judges by individual F values for treatments or by the analysis from the range (*2*), quick method. Rank the judges and use the top 8-12.
   6. Calculate the SNR value, and required tastings to get treatment significance determined from the untested panel performance.
   7. Allow screened judges to evaluate the product the required number of tastings (from No. 6).
   8. Analyze final results of No. 7 by the analysis of variance.
   9. For regional studies the data from at least two laboratories should be combined because of consistent treatment X location interactions in past results.
   10. If the differences indicated by the LSD are not of practical magnitude on the scale used an investigator may wish to determine the number of tastings required for a practical difference (LPD) which could be used for reducing future work with similar panels.

II. Panel of known efficiency or of trained persons
   1. Use 8-12 selected persons for scoring the samples using a form similar to Figure 2.
   2. As a guide for number of replications to present to the judges use the SNR × N from past performances.
   3. Analyze results of No. 2 by analysis of variance.
   4. The data from at least two laboratories should be combined in regional studies.
   5. Same as I-10.

III. Panels which show significant interaction between treatments and judges.
   1. If selected or untested panels show repeatedly significant interaction between treatments and judges, the samples if possible should be presented to a large consumer panel for further testing.

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SWEETNESS OF VARIOUS COMPOUNDS AND ITS MEASUREMENT [a]

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(Manuscript received November 30, 1956)

Sweet substances are noted for their dissimilarity in chemical structure and their marked differences in intensity. The problem of the sweetness of various substances is ever present with the food scientist, especially so when formulation of a product requires substitution of one sweetener for another. The ratio of the sweetnesses of the two products must then be determined. Information in this area is available but from sources using different methods of measurement and different concentrations for comparison. There is definite evidence that the sweetness of one substance relative to another changes, depending on the concentration studied (*2*, *4*).

The studies that have been reported have generally depended on direct comparison of the two stimuli, usually by some variation on the constant stimulus differences techniques. However, many studies have shown that people are quite capable of expressing their judgments of attributes or qualities directly on the basis of an underlying subjective continuum. The purpose of this study was to determine how effectively such an underlying subjective continuum would be related to different concentrations of sweeteners so that the subjective sweetness of various compounds and hence their sweetness relative one to another could be established.

EXPERIMENTAL

The psychophysical method used was that of the single stimulus with a nine-category single-ended rating schedule of intensity. The schedule started with *no intensity* and had subsequent alternate intervals anchored by the terms *slight amount or intensity, moderate amount or intensity, strong amount or intensity* and, finally, *extreme amount or intensity.*

Materials were the purest available: many were C.P. or U.S.P.; other were specially prepared by an organic chemist. Water for preparation of solutions and for mouth rinsing by the subjects was distilled and percolated through charcoal to remove all taste and odor. The subjects did not know what substances they were testing.

Subjects consisted of 15 employees of this Institute. They were given general information on the nature of the study and their task in assigning each stimulus to a point on the rating scale. They were asked not to smoke or eat for one-half hour before a test and were instructed to rinse their mouths with water at the start of a test session and after each stimulus solution. They were also told to take the whole sample, which consisted of 6 ml., hold it in the mouth for a few seconds, and then expectorate.

Preliminary studies using two substances, sucrose and dextrose, in the same session and with the concentrations approximately arithmetically spaced showed that: (a)

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[a] This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 671 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

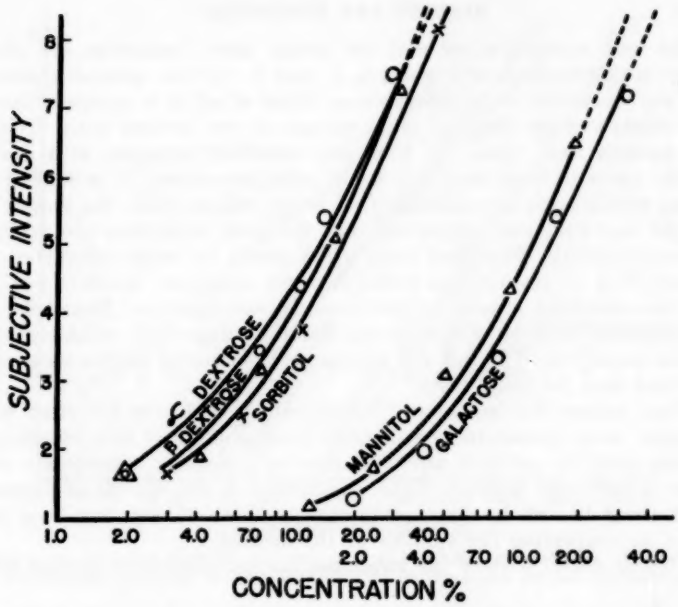

Figure 1. Relation between concentration and subjective intensity. Symbols are actual mean values obtained. Broken lines indicate extrapolation beyond obtained values. (Second line of values on abscissa refer to two compounds on the right of figure.)

mean ratings of subjective intensity plotted against concentration yielded relatively smooth curves; (b) the curves were such that logarithmic spacing should produce nearly straight lines.

For the tests proper, solutions of each substance were prepared in concentrations that were a geometric series with a factor of two; thus sucrose was 2, 4, 8, 16, and 32%. The concentrations were wt. by volume, i.e., g. per 100 ml. of solution. The middle of the series was a concentration that the experimenters judged to be approximately moderately sweet. Exceptions were made for substances that were not sufficiently soluble to fit such a series. In these cases the solubility set the highest concentration but the geometric series was maintained.

Each of 16 substances was measured at a different test session. All sessions were held between 9 and 10 a.m. on Tuesdays and Thursdays. At a session, 10 subjects, drawn as randomly as possible from the pool of 15, participated. Each subject was presented 10 stimuli, two of each of the 5 concentrations, in a random order with a 45-second interval between the time he returned the one-ounce medicine or "shot" glass and the presentation of the next.

The 9 categories of the rating scale were assigned successive integers from 1 to 9 so that the subjective intensity responses could be expressed as numerical scores for analysis. Mean intensity ratings based on 20 judgments for each concentration were obtained, and a least-squares curve was fitted to the means, by applying the method of orthogonal polynomials *(6)*. The assumption was made that the relationship between concentration and subjective intensity is not more complex than one that can be fitted by a quadratic curve. The curve equations were solved for the concentrations of a compound equivalent to mean ratings of intensity from 2 through 8. For comparisons among compounds the ratio of the concentration to sucrose concentration at each of these intensities was computed.

RESULTS AND DISCUSSION

The least squares curve and the actual mean intensities are plotted against concentrations in Figures 1, 2, and 3. Certain general characteristics are observable from these figures. First of all, it is apparent that for most substances the obtained mean ratings do not deviate much from the least squares line. Also, the lines are essentially straight with a slight positive curve in most cases and a few negative curves. It is evident that the log relationship for concentration is one which yields the closest to a straight line function, suggesting that the scale categories are relatively uniform in width. Note that there are 3 points for each concentration of sucrose (Fig. 3) since it was tested on three occasions. Since it was to be used as a standard, a more reliable measure was desirable. Examination of the variation in these points reveals the high degree of reliablity of the method employed. The line for sucrose was computed on the basis of the combined data for the 3 tests.

Mean ratings for lactose and dulcin, which could not be tested within the same range of sweetness as sucrose because of their lack of solubility, indicate that the subjects used the scale in a manner appropriate to the range of sweetness present. Thus, the method is not specific to substances which cover the whole range of intensity. This evidence has been interpreted as supporting the validity of the method.

Figures 4 and 5 show the sweetness curves relative to sucrose for all

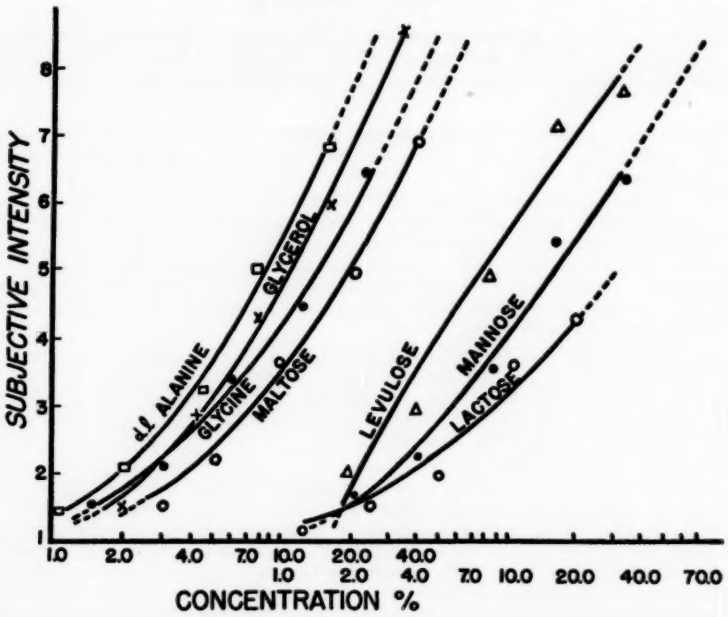

Figure 2. Relation between concentration and subjective intensity. Symbols are actual mean values obtained. Broken lines indicate extrapolation beyond obtained values. (Second line of values on abscissa refer to three compounds on the right of figure.)

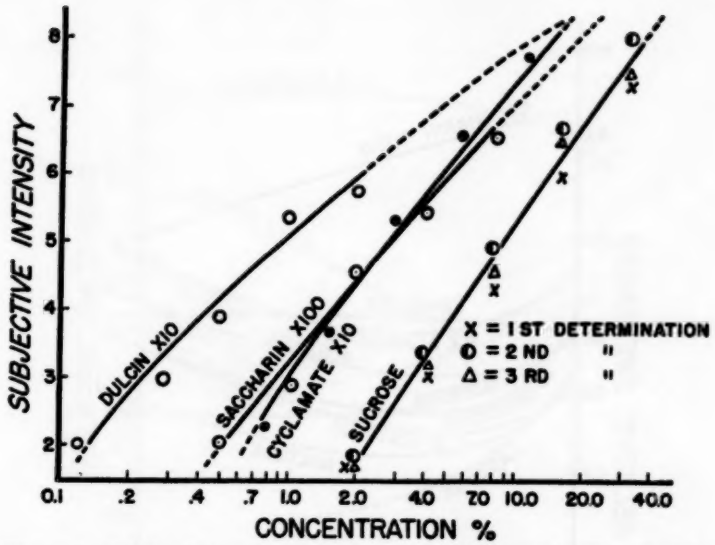

Figure 3. Relation between concentration and subjective intensity. Symbols are actual mean values obtained. Broken lines indicate extrapolation beyond obtained values.

the compounds, for intensities 2 through 8. It is immediately apparent that at first relative sweetness decreases slightly and then increases with increasing concentration. Exceptions to this tendency are levulose which has a fairly constant relative sweetness and the "artificial" sweeteners of entirely different chemical structure, dulcin, saccharin and calcium cyclamate which all decrease in relative sweetness with increasing concentration. The rationale for various types of relative sweetness curves is not easy to develop. Why some substances should become less sweet relative to sucrose as concentration changes while others become more sweet is perplexing. Certain hypotheses can be advanced to explain what may at least contribute to this effect. One possibility is that differences in structure produce differences in diffusion into the receptors. In the case of those compounds which become relatively less sweet there is the possibility that other qualities, especially bitter, may mask the sweetness. Subjective reports of bitter, particularly at the higher concentrations, give some support to this idea but these hypotheses do not explain the results for levulose and sucrose vs. the other related compounds. Changes in relative sweetness with concentration for a number of compounds have been studied by Cameron *(2)* and Dahlberg and Penczek *(4)*. Their results show the same tendency for many substances to become relatively more sweet with respect to sucrose with increasing concentration. However, if glucose were chosen as the standard then most of the natural compounds would exhibit a straight line for the relative sweetness curves and sucrose and levulose would stand out as exceptions.

Figure 1 also reveals another interesting fact. Two forms of *d* dextrose were tested, α and $\alpha\beta$. The $\alpha\beta$ form is the equilibrium mixture

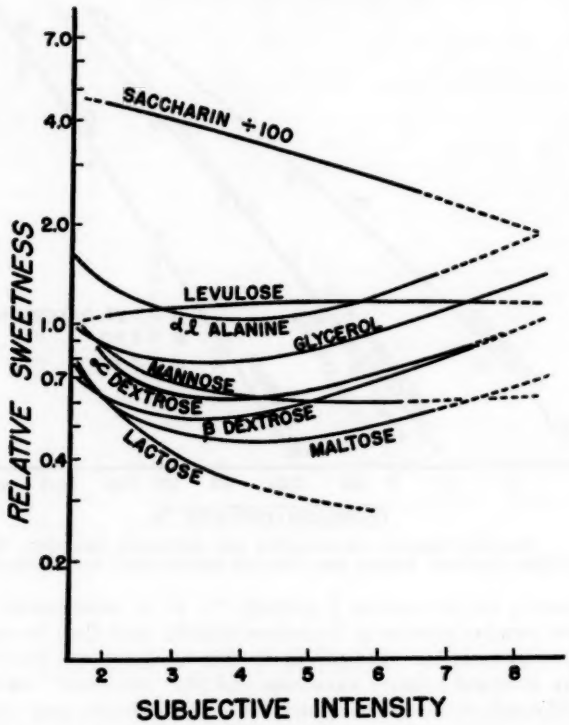

Figure 4. Relative sweetness compared to sucrose at different subjective intensities. Broken lines indicate extrapolation beyond obtained values.

which forms upon standing. Cameron *(3)* did some pilot work which indicated that the α form is the sweeter. This result is borne out by our results and can be seen in the divergence of the two dextrose curves. Note that they merge at the higher intensities.

Table 1 gives the relative sweetness values for each substance at a moderate level of sweetness (5 on the 9 category scale). This represents a subjectively common point to compare the various compounds. Sucrose at this intensity is a 9.12% concentration. The values for these compounds found by other experimenters are also given. Whenever possible the value was chosen which represents a comparison with a 10% sucrose solution. In order to give an idea of the values obtained when comparisons are made by determining absolute thresholds, several of this type are included.

Examination of Table 1 shows that the pattern of results obtained in the present experiment is comparable to that obtained by other experimenters. The actual values are in many cases very close even though the psychophysical methods used were different. The order for other levels of intensity are very much the same as can be observed by examination of Figures 4 and 5. In most cases the curves do not cross each other.

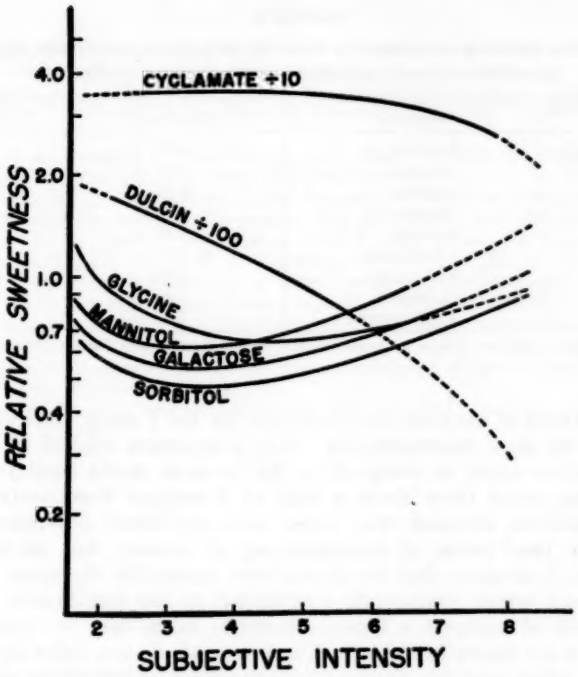

Figure 5. Relative sweetness compared to sucrose at different subjective intensities. Broken lines indicate extrapolation beyond obtained values.

TABLE 1

Sweetness relative to sucrose at moderate intensity (9.12% sucrose)

Substance	Schutz-Pilgrim	Other investigators [1]
Saccharin	306.0	675 (7)[2]
Dulcin	90.7	265 (7)[2]
Ca. Cyclamate	33.8	
Levulose	1.15	1.15 (4), 1.16 (2), 1.75 (1)[3]
DL-Alanine	1.07	.90 (10)
Glycerol	.84	.77 (2), 1.08 (3)[3]
Mannitol	.72	.62 (3)[3]
Glycine	.65	.62 (10)
α-Dextrose	.64	
α,β-Dextrose	.61	.79 (4), .68 (2), .65 (8), .76 (9), .74 (1)[3]
Galactose	.59	.67 (2), .32 (1)[3]
D-Mannose	.59	
Sorbitol	.51	.54 (3)[3]
Maltose	.46	.47 (4), .32 (1)[3]
Lactose	.30	.48 (4),[4] .38 (2), .16 (1)[3]

[1] Compared with 10% sucrose unless otherwise noted.
[2] Compared with 2% sucrose.
[3] Ratio of concentrations at absolute threshold.
[4] β-Lactose.

TABLE 2

Mean values for seven compounds in order of subjective intensity and significant differences between compounds at 1% level of significance

Compound	Mean value [1]
Levulose	4.95
Sucrose	4.92
Sucrose	4.70
Glycerol	4.58
Sucrose	4.49
α-Dextrose	4.07
D-Mannose	3.86
Galactose	3.84
α,β-Dextrose	3.83

[1] The values contained within the same bracket are not significantly different from one another.

An analysis of variance was conducted for the 7 compounds which were tested at the same concentrations. Only compounds studied at the same concentrations could be analyzed in the variance model employed. Since sucrose was tested three times a total of 9 sessions were analyzed. The over-all analysis revealed that there were significant differences among compounds (and levels of concentration, of course), but no significant interaction, indicating that the slopes were essentially the same. In order to determine which compounds contributed to the significance found in the analysis of variance a Duncan multiple range test *(5)* was applied. The results are shown in Table 2. The 3 sucroses do not differ significantly from each other, and this fact can be interpreted as indicating the reliability of the method although it is recognized that compound is confounded with session. However, if a sensitive test of the relative intensity of 2 compounds is required, they should both be rated in the same session by the same subjects. In addition, the concentration-subjective intensity relationship was tested for quadratic curvature. This curvature can be seen visually in Figures 1, 2, and 3. Glycerol, galactose, α-dextrose and α,β-dextrose were significantly curved. If the values for these compounds are used as a guide it can be estimated that of the compounds not included in the analysis, sorbitol, mannitol, and DL-alanine also have significant curvature.

SUMMARY

Sixteen compounds were rated on a subjective intensity scale for sweetness at each of five concentrations. The method of single stimuli using this subjective intensity scale yields results which are meaningful and reliable and which are more easily obtained than the more traditional methods such as paired comparisons. The relationship between subjective sweetness and concentration was established. It is basically logarithmic. The ratios of the concentration of each of 15 of the compounds to the concentration of sucrose of the same subjective intensities were computed to determine their relative sweetnesses. As subjective intensity or concentration increases relative sweetness varies in one of three patterns depending on the compound.

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IDENTIFICATION OF THE PINK PIGMENT OF CANNED TUNA[a]

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(Manuscript received December 1, 1956)

In the commercial processing of tuna the whole fish is precooked after evisceration prior to cleaning, sorting, and packing in cans for heat sterilization. A pink pigmentation is characteristic of good precooked tuna flesh. This pink pigment is stable during thermal processing and is one of the quality attributes of canned tuna. The pigment responsible for this normal and desirable pink color of canned tuna has not been described. Knowledge of the chemical nature of this pigment would be of considerable basic importance in the studies of the discoloration of canned tuna. There is, additionally, the possibility that the pigment is not unique to tuna but is of more widespread occurrence and therefore of general interest. Results of experiments leading to the identification of this pink pigment of tuna are presented in this report.

EXPERIMENTAL

Tuna. Frozen yellowfin tuna *(Neothunnus macropterus)* and commercially precooked loins were obtained through the courtesy of the F. E. Booth Packing Company, San Francisco. The whole tuna and the precooked loins were held in frozen storage until use. A variety of canned tuna samples was purchased from local retail markets.

Reflectance spectra. Reflectance spectra were recorded with a Beckman DR spectrophotometer equipped with a reflectance attachment. Usually, an aqueous solution of the reducing agent, sodium hydrosulfite, was painted on the surface of the cooked tuna prior to measurement of reflectance spectra.

Hemochromes. In studying the possibility of heat formation of denatured globin hemochrome, powdered cattle hemoglobin was dissolved in phosphate buffer at pH 7.0 to make a 0.002 M. solution. A series of tubes containing 3.0 ml. of such solutions were placed in a boiling water bath. Tubes were removed from the bath at 5- to 30-minute intervals. The precipitated material was separated by filtration and washed three times with distilled water, then placed in 10 ml. of a 10 M. urea solution at room temperature, where it was allowed to stand with periodic agitation for a period of time, usually overnight, to effect solution of most of the precipitate. The urea solutions were then filtered and absorption spectra were recorded with a Beckman DR spectrophotometer.

The model system used in testing hemochrome formation in these experiments consisted of 0.4 ml. of a 0.80 M. solution of non-heme substance (nicotinamide, choline chloride, betaine, histidine hydrochloride, diphosopyridine nucleotide, sodium nitrite), 0.4 ml. of a 0.0023 M. solution of hematin (crystalline hemin in 0.1 N. NaOH), 2.0 ml. of 100 mg./ml. solutions of reducing agents (sodium sulfite, ascorbic acid, sodium ascorbate, glutathione, cysteine · HCl), and water to a total volume of 5.0 ml. The only variation in this system occurred when sodium hydrosulfite was used as a reducing

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[b] Chemist, Fish and Wildlife Service.

agent or when nicotinic acid was used as the non-heme compound, in which cases these crystalline substances were added in powdered form to final concentrations equal to those indicated previously.

Laboratory heat treatments. In precooking tuna in the laboratory, samples of 100 g. of frozen tuna loin were taken, thawed, then ground in a mortar and pestle with additions, where indicated, of 200 mg. of nicotinamide or 0.1 N. NaOH solution to a pH of at least 8. The 100-g. portions were then shaped into patties, wrapped securely in aluminum foil and cooked over boiling water for 30 min. Chicken-breast samples were similarly treated.

For studies of methods of restoring color, small pieces (1- to 2-cm. flakes) of commercially precooked loins were first exposed to air until complete loss of pink color occurred. Then 10 g. were loosely packed in clear glass vials approximately 25 x 95 mm. in size, and hot, freshly boiled distilled H_2O was added. Additions, where incorporated, consisted of nicotinamide in 0.05, 0.10, and 1.0% levels, either alone or in combination with levels of 0.10 to 0.10% of sodium ascorbate, cysteine, and sodium hydrosulfite. The filled vials were tightly screwcapped and cooked at 10 p.s.i. for 15 min.

RESULTS AND DISCUSSION

General properties. An immediately apparent property of the pigment is its pink color. This color appears in precooked and canned tuna; hence, it is quite heat stable. The exposure to air of pink precooked or canned tuna results in a relatively rapid change of the pink color to tan. That this change is oxidation may be demonstrated by adding to the faded (tan) surface a reducing agent such as sodium hydrosulfite, in which case the pink color immediately returns.

The pink color is not visibly affected by dilute hydrochloric acid or sodium hydroxide. The pigment is not soluble in water, acetone-ether, petroleum ether, NH_3-NH_4Cl buffer (pH 9.0), chloroform, dilute hydrochloric acid, glacial acetic acid, or pyridine.

The principal known pigments of tuna flesh are hematin compounds. Matsuura and Hashimoto *(8)* report that ordinary muscle, i.e., tuna loin, of yellowfin tuna *(Neothunnus macropterus)* contains a total of 140 mg. per cent hemoglobin and myoglobin. Our knowledge of the chemistry of the hematin compounds *(7)*, when considered with the aforementioned properties of the pigment, suggests that the pink color is that of a hemochrome.

Direct spectral measurements. The spectral properties of hematin compounds in general and, in particular, the hemochromes are highly characteristic and quite distinct *(7)*. Advantage was taken of this fact in affirming the relation of hemochromes to the pink color of tuna. The insolubility of the pigment prevented the obtaining of absorption spectra; however, it was possible to obtain good reflectance spectra (Figure 1) on a large number of samples, including commercially precooked tuna, laboratory precooked tuna, and commercially canned tuna. Since the color readily oxidizes to tan in air, these measurements were made on samples reduced with sodium hydrosulfite. Similar reflectance spectra of untreated, freshly cut, pink surfaces of precooked or canned tuna can be obtained if the measurements are rapidly made before oxidation occurs; therefore, the addition of the reducing agent does not result in any alteration of the pigment or in the formation of any new compound. A reflectance spectrum of an untreated sample of commercially canned tuna is included in Figure 1.

The reflectance minima are the same in all cases, indicating that the same pigment is responsible for pink color in canned and precooked tuna. These highly characteristic spectral curves, with reflectance minima at about 555, 530, and 415 mμ, are exactly those to be expected for hemochromes *(7)*.

Identity of the hemochrome. Myoglobin and hemoglobin as the principal hematin compounds in tuna loin are certainly the contributors of the heme portion of the hemochrome molecule. There remains, then, the problem of identifying the nitrogenous compound constituting the other portion of the molecule. It is difficult to distinguish between different hemochromes

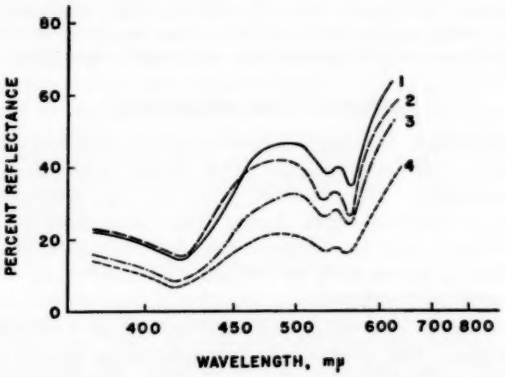

Fig. 1. Reflectance spectra of tuna preparations. 1. Commercially precooked tuna. 2. Laboratory precooked tuna. 3. Commercially canned tuna. Samples used for 1, 2, and 3 were reduced with sodium hydrosulfite. 4. Commercially precooked tuna, unreduced.

by spectral measurements; the reflectance spectra shown in Figure 1, for example, are characteristic for many such compounds. Hence, another approach to the problem was necessary. Since the desirable pink color of good precooked tuna is not localized but is distributed throughout the loin, it is necessary for the hemochrome-forming nitrogenous compound to be distributed throughout the tuna. Additionally, it must occur in fairly large amounts and be able to react to form a hemochrome. There are at least two compounds which would react in this manner. First, since protein denaturation will occur during precooking of tuna, it is probable that denatured globin will interact with a heme nucleus to form denatured globin hemochrome. Previous investigators have prepared denatured globin hemochrome by dissolving hemoglobin in alkaline solutions of high pH value, e.g., pH 10 *(1, 4, 6, 9)*. Since such high pH values do not exist in highly buffered tuna flesh it is necessary to postulate that heat, at a more nearly neutral pH, serves as an agent in the formation of denatured globin hemochrome. It is difficult to test such a postulate directly, since denatured globin hemochrome is most soluble in alkaline solutions and such solutions must be avoided. An indirect approach, in which heat-denatured hemoglobin has been put into solution by concentrated urea solution, has been used. Absorption spectra for such solutions are given in Figure 2.

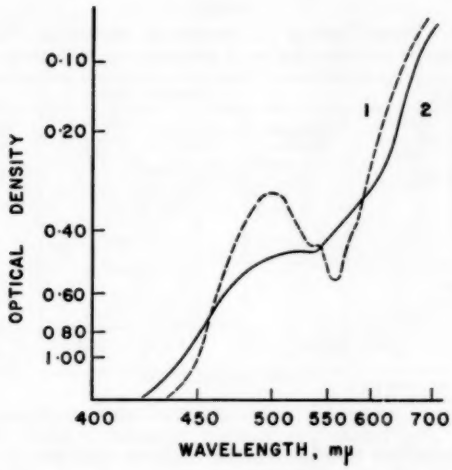

Fig. 2. Absorption spectra of heat-denatured hemoglobin in 10 M. urea solution. 1. Sample reduced with sodium hydrosulfite. 2 Sample unreduced.

The characteristics of these spectra (Figure 2) with a maximum at 555 mμ and a deflection at 530 mμ indicate the presence of denatured globin hemochrome; the curve for the unreduced sample is likewise that expected for the oxidized denatured globin hemochrome. This indicates that denatured globin hemochrome may be formed by heat under non-alkaline conditions.

A second substance which might be expected to react to form a hemochrome is nicotinamide, or its equivalent, which is present in tuna in amounts of approximately 15 mg. % *(11)*. Considerations of this possibility, and others, have been treated primarily in model systems.

Since hemochromes and related compounds have highly characteristic absorption spectra, it is convenient to study their formation by spectrophotometric means. Such studies on model systems, utilizing either aqueous extracts of tuna flesh or heme proteins such as hemoglobin, were complicated, in general, by persistent turbidity and were, accordingly, unsatisfactory. However, systems using crystalline hemin as the hematin compound were very satisfactory to use. With such a system, a number of nitrogenous compounds were tested for their ability to form hemochromes; since hemochromes are readily oxidized to hemichromes it is necessary to afford reducing conditions in the model as well as in tuna; hence, a number of reducing agents were also tested. The extent of hemochrome formation by different couples of nitrogenous compound and reducing agent is indicated in Table 1.

These results indicate that nicotinamide will readily form a hemochrome with heme under reducing conditions, in either neutral or basic solution, and furthermore, that nicotinamide is superior in terms of hemochrome-forming ability to any other substance tested. The spectra obtained were always typical of hemochromes except in the case of the nitrite sys-

TABLE 1

Visual ratings of hemochrome formation
(Plus sign(s) indicates degree of pinkness over unreduced blank)

Non-heme constituent	Reducing Agent					
	Sodium Hydrosulfite	Sodium Sulfite	Ascorbic Acid	Sodium Ascorbate	Glutathione	Cysteine
Nicotinamide	++++[1]	−	+	++[1]	+	+
Nicotinic Acid						
Buffered[2]	++[1]	−	−	±	±[1]	−
Unbuffered	−	−	−	−	−	−
DPN	±			±		
Choline	−[1]					
Betaine	−[1]					
Histidine	−[1]					
Nitrite	+[1]	−	+		++[1]	+++

[1] Absorption spectra obtained
[2] Phosphate buffer, pH 7.0

tems, where absorption spectra showed diffuse maxima at about 540 and 565 mμ. The most effective reducing agent tested was sodium hydrosulfite; however, the sulfhydryl compounds were reasonably effective and it seems likely that in tuna flesh it is this type of compound that is responsible for maintenance of reducing conditions.

Diphosphopyridine nucleotide proved to be far less effective than free nicotinamide in reacting with heme to form a hemochrome in this model. Since nicotinamide must occur primarily in combined form in tuna, and since the free form is necessary for effective hemochrome formation, there must occur diphosphopyridine nucleotide breakdown. The presence of diphosphopyridine nucleotide nucleosidase could not be detected in three different tuna samples; however, it is known that nicotinamide is split quantitatively from diphosphopyridine nucleotide by autoclaving at 120° C. at pH 6.8 for 7 minutes *(10)*. This heat lability was confirmed. Thus, the availability of nicotinamide may be accounted for by the thermal treatment accorded tuna in the precook.

In some small-scale laboratory cooks of ground raw yellowfin tuna it was found that the addition of nicotinamide prior to cooking resulted in a pinker "precooked" product. It was, however, difficult to prepare such small samples and cook them under reducing conditions. The hemochrome resulting in the product was inevitably oxidized on the surface and the degree of pinkness could best be ascertained by viewing a freshly cut interior surface or by surface reducing with sodium hydrosulfite. In similar cooks alkali was added to change the pH of the flesh. This did not affect the resulting hemochrome formation as judged by pinkness. A reflectance spectrum of cooked yellowfin tuna loin containing added nicotinamide is included in Figure 3. For purposes of comparison, similar cooks with added nicotinamide were made with chicken-breast muscle. Reflectance spectra were obtained and a typical example appears in Figure 3.

It is clear from comparing spectra in Figure 3 with those in Figure 1 that the same compound, namely, a hemochrome, is responsible for the characteristic curves.

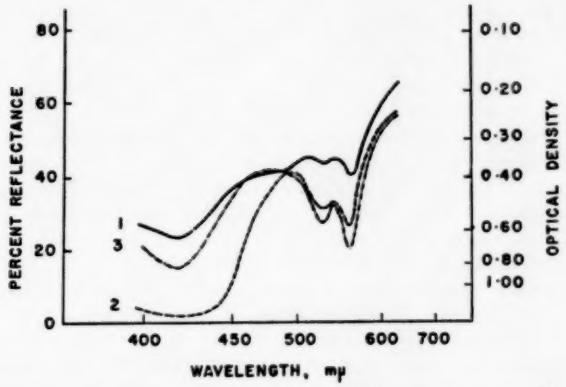

Figure 3. Hemochrome reflectance and absorption spectra. 1. Chicken-breast sample, reflectance. 2. Tuna, reflectance. 1 and 2 were cooked with added nicotinamide and were reduced. 3. Absorption spectra of nicotinamide hemochrome.

Stabilizing the pigment of canned tuna. Some preliminary studies indicate that it may be possible by adding nicotinamide and, perhaps, reducing agents, to improve color quality in canned tuna by stabilizing the pink pigment. In these experiments small samples of commercially precooked tuna with or without additives were heat-processed in sealed glass vials and the pink color present was visually compared to control samples. It was difficult to obtain completely objective evidence in these small-scale studies, as it was found that variations in the tuna samples selected were of great importance. That is, if samples from good precooked loins were used, then the color in all the packs, including controls, was satisfactory. If the good loins were subjected to oxidative deterioration, then certain additives were helpful in improving color; but reducing conditions in the cook were more important.

In general, it was found that the addition of nicotinamide and maintenance of reducing conditions during the cook resulted in color improvement. The addition of 0.05% nicotinamide exerted a definite color improvement in the samples of poor color quality; color improvement was also noticed when 0.05% sodium ascorbate was added alone, or better, together with nicotinamide. These compounds added in the levels indicated here would almost certainly satisfactorily improve color in commercial packs; the testing of considerably lower levels, e.g. 0.01%, under the more favorable industrial conditions is warranted.

CONCLUSIONS

On the basis of the general properties of the pigment and the highly characteristic and consistent spectral properties of precooked and canned tuna, laboratory samples, and model systems, it is concluded that the pink pigment of canned tuna is a hemochrome. It is clear that the two most likely hemochromes have as their non-heme constituent either denatured globin or nicotinamide or, perhaps, both. It is difficult to distinguish be-

tween two such hemochromes because of the extreme similarity of their spectral properties; however, it is known *(2, 3, 5)* that denatured globin has a very high affinity for heme and, therefore, it would be expected to be involved. The insolubility of the pigment supports the conclusion that the hemochrome contains denatured globin. However, it has been demonstrated herein that nicotinamide also readily forms a hemochrome with heme; furthermore, nicotinamide or its precursors occur in large quantity in tuna, and, under the conditions of tuna processing, free nicotinamide would be available. It is then concluded that both nicotinamide and denatured globin hemochrome may occur in tuna. Since two molecules of non-heme substance combine with each heme molecule, mixed nicotinamide-denatured globin hemochromes probably occur. From the standpoint of practical industrial considerations there is little difference, as each of the three possible hemochromes would contribute the desirable pink color.

The similarity of reflectance spectra of cooked chicken-breast muscle to those of tuna products indicates that the contribution of hemochrome compounds to the color of cooked meats may be of a more general nature. General studies of the pigments of cooked meats have been made and will appear separately.

Stabilization of the pink pigment of canned tuna by the addition of nicotinamide and reducing agents such as ascorbic acid to canned tuna, deserves the consideration of the industry. The addition of hemochrome-forming compounds to canned tuna would appear more promising in providing uniformity of tuna color rather than improving color in badly deteriorated samples. The extreme ease of oxidation of the pink hemochrome to the less desirable tan hemochrome makes it imperative that reducing conditions prevail in tuna processing, if a product of good color quality is to result.

SUMMARY

Studies of the properties of the pigment responsible for the desirable pink color of precooked and canned tuna show that the pigment is a hemochrome. Spectral studies of commercial tuna preparations, laboratory preparations, and model systems confirm the nature of the pigment. Furthermore, the results indicate that the hemochrome is derived from the reaction of heme of myoglobin and residual hemoglobin with either denatured globin or nicotinamide or both. This pink pigment in canned tuna may be stabilized by the proper addition of hemochrome-forming compounds and/or reducing agents.

Acknowledgments

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A CHROMATOGRAPHIC STUDY OF SOME OF THE COMPOUNDS IN ROASTED COFFEE

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(Manuscript received December 3, 1956)

Flavor is the most important factor in coffee from the standpoint of the consumer. It is known that the green coffee itself, its handling, manner of roasting, grinding, storage, method of brewing and handling after brewing, all affect flavor; yet, an understanding of the chemical nature of flavor and changes in flavor of coffee remains to be found. It was thought that some of the newer chromatographic techniques might be adapted to a study of compounds which might be important in coffee flavor and helpful in determining which substances are native to coffee and which are produced in roasting. There is also the possibility that information on the nature of staling, on flavor changes in boiling brewed coffee, and on some of the more subtle changes in flavor might be found. The methods employed indicated many compounds which have not been identified as yet; some of these are native to coffee and some are produced by roasting. Although some of these compounds are not yet even tentatively identified, the reproducibility and the characteristic nature of the chromatograms indicated the value of the methods developed. Also, the techniques appeared to be applicable to other food. Consequently, this report does not await complete identification of all unknown substances observed.

EXPERIMENTAL

Non-volatile acids and phenols

Materials. *Roasted Coffee.* Unless otherwise specified, the analyses were performed on that coffee obtained from The Coffee Brewing Institute, Inc., and hereafter referred to as *standard* coffee. Other samples were purchased locally.

Green coffee: A ground sample of Santos coffee was used.

Chromatographic solvents: Dimethylformamide, cyclohexane, and t-butyl alcohol were technical grades. For heptane, Skellysolve-C was used. All other solvents were reagent grade. None of the solvents was further purified.

Procedures. *Preparation of extracts*: A series of extracts was prepared as follows: Distilled water (500 ml.) was added to ground roasted coffee (100 g.) and the mixture was placed in a refrigerator (2° C.) for 2-3 days. The grounds were removed by filtration, and a second extraction of the same grounds was carried out at 85-90° C. The grounds were again removed and finally extracted with boiling water. Ecah of the above 3 extracts was divided into 2 portions, one of which was acidified and the other made alkaline. The 6 extracts were finally concentrated to syrups by distillation under reduced pressure. In addition to these extracts, acidic and alkaline concentrates of green coffee (extracted at 85-90° C.) were prepared.

Paper chromatography. The chromatographic procedure and indicators were essentially those of Buch *et al.* (3), with *iso*-amyl alcohol (rather than *n*-amyl alcohol) constituting the moving solvent.

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Extracts (as well as known compounds) were spotted on Whatman No. 1 filter paper, 8 x 8 inches, by means of a cut-off No. 22 hypodermic needle to give spots approximately 2 mm. in diameter. Best results were obtained by applying each extract to its respective spot a total of 8-10 times to effect concentration. The paper was then stapled in the form of a cylinder, suspended 12 hours in an atmosphere of 5 M. formic acid equilibrated against *iso*-amyl alcohol, and finally developed for 6-8 hours with *iso*-amyl alcohol equilibrated against 5 M. formic acid. The paper was dried at room temperature for 12 hours and sprayed with the appropriate indicator.

RESULTS AND DISCUSSION

The results (tabulated in Table 1) show the presence of at least 10 acidic constituents. All of the extracts noted above, as well as a solution of commercially available soluble coffee, gave essentially the same qualitative pattern. Apparently, cold-water extraction is sufficient for such a study. Further extraction with hot water gave extracts which contained

TABLE 1

Paper chromatography of non-volatile acids and phenols in roasted coffee

Solvent: *iso*-Amy alcohol saturated with 5 M formic acid.

Material	Spot	Rf	Indicator		
			Ultra violet Fluorescence	$FeCl_3$ (3%)	Bromphenol Blue
Coffee extract:					
	(1)	0.82	×		
	(2)	.70	×		
	(3)	.56	×	×	×
	(4)	.44	×	×	×
	(5)	.32	×	×	×
	(6)	.22			×
	(7)	.10		×	×
	(8)	.07	×	×	×
	(9)	.05		×	×
	(10)	.01	×	×	×
Caffeic acid:		.70	×	×	×
Chlorogenic acid:		.55	×	×	×
Quinic acid:		.09		×	×
Riboflavin:		.07	×		

the same constituents, but in much lower concentrations. Furthermore, the latter extracts contained large mounts of undesirable, acid-precipitated material.

Seven of the 10 spots showed varying degrees of fluorescence under ultraviolet light. Most of the spots showed an acidic reaction with bromphenol blue, and all of them gave positive reactions with a sensitive ammoniacal silver nitrate reagent. At least 6 of the constituents were indicated to be phenolic by reaction with ferric chloride.

All of these compounds appeared in the extract of green coffee and apparently are native to the coffee bean rather than being pyrolytic products produced by roasting. Only 4 known compounds were chromatographed; they were caffeic acid, quinic acid, chlorogenic acid, and riboflavin, and the R_f values of these compounds correspond to four of those

appearing in coffee. In general, spots numbered 3, 4, 5, and 8 in Table 1 predominate on every chromatogram when viewed under ultraviolet light. Spot No. 3 appears to be that of chlorogenic acid. Spot No. 2 corresponds to that of caffeic acid and was generally of very low intensity. Extracts which were boiled with hydrochloric acid failed to produce spots corresponding to chlorogenic acid and two of the unidentified compounds, spots No. 4 and No. 5; whereas, the spot in the position of caffeic acid was greatly intensified. It appears, therefore, that isomers of chlorogenic acid are involved.

Volatile Acids

Organic acids present in roasted coffee might be expected to contribute to the flavor of the brewed coffee, principally because of their acidic character. The presence of volatile acids, however, would be of particular significance, since these acids exhibit characteristic flavors in addition to their acidic properties. In preliminary experiments the odor of lower fatty acids was noted in the distillate when acidified coffee brew was concentrated under vacuum. This observation suggested that these acids were present in concentrations sufficient to contribute to the characteristic coffee aroma.

The organic acids in roasted coffee have been studied by Mabrouk and Deatherage (*8*), who used an acidic silica-gel column. Such a column does not normally separate the volatile acids; however, a buffered column provides an excellent means of separating these acids, and for this study the method of Harper (*5*) was applied.

EXPERIMENTAL

Preparation of extracts. Extracts of standard coffee, 2 commercial brands of roasted coffee, and of green coffee were prepared as follows: Freshly opened coffee (100 g.) was mixed with 10 ml. 0.5 N. sulfuric acid in a mortar and extracted with ether in a Soxhlet extractor for 24 hours. The thimble was removed, and the ether was evaporated from the grounds. The latter were mixed with an additional 40 ml. of the sulfuric acid and extracted 36 hours with the ether remaining from the first extraction. The ether was then extracted with just sufficient 0.1 N. sodium hydroxide to neutralize the acids (as indicated with phenol red), and the aqueous extract was filtered, evaporated to 30-40 ml., and made up to 50 ml.

Chromatographic procedure. Ten grams of washed silicic acid was mixed with 10 ml. 2 M. pH 7.2 phosphate buffer in a mortar. The mixture was suspended in washed chloroform and packed in a column 3 cm. in diameter. An aliquot of coffee extract representing 2-10 g. coffee was acidified with 0.5 N. sulfuric acid, taken up on silicic acid, and packed above the column. The acids were eluted with mixtures of chloroform and *n*-butanol in the following sequence: 80 ml. 0.5%, 80 ml. 1%, 80 ml. 5%, 100 ml. 10%, and 100 ml. 50%. Fractions of 4 ml. were collected, using an Autonomos collector, and titrated with carbonate-free 0.01 N. sodium hydroxide using phenol red as indicator.

Mixtures of various combinations of known acids were separated in the same way, and positions of the peaks were compared.

RESULTS AND DISCUSSION

The 3 samples of roasted coffee gave titration curves which were substantially the same, and a typical curve from standard coffee is illustrated in Figure 1. Five acids appeared in significant amounts, and by comparison with the titration curves of known acids four of these have been identi-

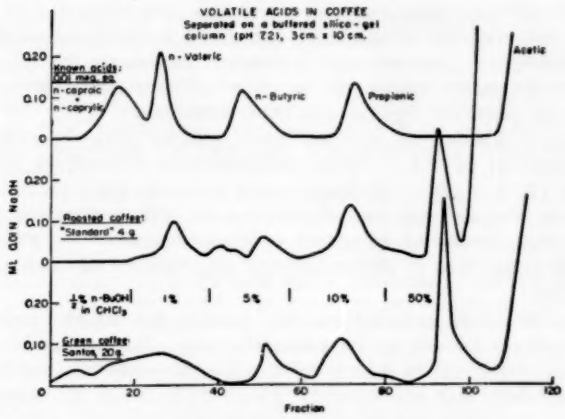

Figure 1. Typical titration curves from roasted and from green "standard" coffee compared with curve for volatile acids.

fied as acetic, propionic, butyric, and valeric acids, respectively. The fifth acid does not correspond to any of the known acids which were studied and probably represents a substituted acid. Acetic acid appears to predominate.

Attempts to verify the identities by means of paper chromatography gave inconclusive results. However, the respective peaks exhibited odors which were in agreement with the tentative identities. The same peaks were obtained when the neutralized fractions from 4 separations of standard coffee were combined, evaporated, and rechromatographed, and the latter peaks were more sharply defined.

Separations of standard coffee indicate the following composition expressed as per cent of roasted coffee: propionic acid, 0.02%; butyric acid, 0.01%; and valeric acid, 0.02%. Acetic acid appeared to constitute more than 0.3% of the coffee, but no attempt was made to elute the latter acid completely.

It is significant that green coffee produced a titration curve (Figure 1) which was very similar to that of roasted coffee. The unidentified acid is somewhat more prominent, however, and appears to be native to the coffee bean and is not a product of pyrolysis.

The data indicate that certain volatile acids are characteristically present in coffee. Although these acids constitute a relatively small share of the total acidity, they may contribute much to the aroma of coffee. Moreover, by virtue of their volatility, they may be involved in the changes which occur during brewing processes. A comparative study of various types of roasts and brews would be of interest.

The Carbonyl Compounds

Any study pertaining to flavor must eventually include the carbonyl compounds, but the separation and identification of these compounds present many problems. This is especially so in the case of roasted coffee, which

represents a complex plant material which has been subjected to relatively high temperatures. In addition to the great number of aliphatic and aromatic aldehydes, ketones, and keto-acids commonly found in native plant materials, many others can be expected as a result of pyrolysis of carbohydrates, proteins, lignins, and other fractions.

Recently, a number of workers have applied paper chromatography to the separation of 2,4-dinitrophenylhydrazine derivatives of carbonyl compounds (*1, 4, 7, 10*). Although such methods have limitations, they are relatively simple, and lend themselves to both qualitative and quantitative determinations by means of spectrophotometry (*4, 6*). The purpose of this study was to determine the applicability of such methods to roasted coffee.

In general, those chromatographic procedures which were used by previous workers proved to be unsatisfactory. Roasted coffee appeared to yield many derivatives which were not sufficiently soluble in the conventional solvents, and consequently, streaking and low R_f values resulted. New solvent systems were, therefore, developed.

The preparation of suitable extracts presented other difficutlies. Hot water extracted much colored material which interfered with chromatography. Ether extracted large quantities of lipid material which was carried through with the neutral derivatives. Cold-water extracts, however, appeared to be satisfactory for this preliminary qualitative study.

EXPERIMENTAL

Preparation of 2,4-dinitrophenylhydrazine derivatives. Two hundred grams of freshly opened coffee was suspended in 1.5 liters of distilled water and the mixture was allowed to stand for 48 hours at 2° C., with occasional shaking. The mixture was filtered while cold, and the filtrate was mixed with 200 ml. of 6 N hydrochloric acid saturated with 2,4-dinitrophenylhydrazine. After 4 hours at room temperature the mixture was extracted with five 75-ml. portions of chloroform. The extracts were combined, filtered, evaporated to 150 ml., and transferred to a separatory funnel. Cold 1% aqueous sodium carbonate was then added in small portions with shaking until the aqueous layer was just alkaline to thymol blue (spot plate), and the layers were separated. The chloroform layer was washed with water, fiiltered, and evaporated to dryness in an air stream to leave the neutral carbonyl derivatives. The aqueus carbonate layer was acidified with 1 N hydrochloric acid and extracted with three 50-ml. portions of chloroform. The extracts were combined, washed, filtered, and evaporated to leave the acidic carbonyl derivatives. The preparations were finally dried over calcium chloride.

Derivatives of the carbonyl constituents of green coffee were prepared in a similar manner. However, a hot-water extract (85°-90° C.) was used, and this was acidified and filtered so as to remove acid-precipitable matter before addition of the 2,4-dinitropheylhydrazine reagent. To avoid emulsions a continuous ether extraction was used to extract the derivatives.

Procedure. *Chromatographic procedure, neutral carbonyl derivatives*: The neutral carbonyl derivatives were dissolved in a minimum amount of 1:1 mixture of chloroform and dimethylformamide, and the solution was applied to Whatman No. 1 paper with a cut-off No. 22 hypodermic needle as a streak, approximately 1.5 mm X 10 cm. Derivatives of known compounds were applied as spots, approximately 1.5 mm. in diameter. Various sizes of paper were used, but in general, Rf values were determined on small sheets 8 X 8 inches. For simultaneous comparison of unknowns with knowns, various combinations were applied to paper tape 1 X 18 inches and the solvents were allowed to move greater distances and in some instances off the paper.

Uni-dimensional chromatograms were developed with a 4 different solvents by ascending and combination ascending-descending techniques as described below. Spots

and bands were detected by the observation of colored areas in visible light, by observation under ultraviolet light, and by colors produced in an atmosphere of ammonia. In the latter method, the chromatograms were exposed to ammonia immediately after removal from the chromatographic chamber, or the dry chromatograms were steamed, and then lowered into a jar containing a few ml. of concentrated ammonium hydroxide.

Solvent I: The chromatograms were equilibrated in an atmosphere of methanol saturated with heptane and developed with heptane saturated with methanol as described by Bassett (*1*).

Solvent II: This method was developed independently, but was later found to be similar to that of Tarbell (*10*), as applied by Buyske *et al.* (*4*). Cyclohexane and dimethylformamide were shaken together in a separatory funnel, and after the mixture had returned to room temperature it was again shaken and allowed to separate. Most of the lower layer was drained into a small beaker containing an inverted Soxhlet thimble (40 X 130 mm.), and the beaker was placed in the cylindrical chromatographic chamber. The upper layer, along with 2-3 ml. of the lower layer, was drained into the chamber itself. The chromatograms were first suspended for one hour in a separate cylinder containing 20-30 ml. of dimethylformamide at 100° C. (over steam). They were then transferred to the chromatographic chamber for 4 hours for equilibration, and finally lowered into the cyclohexane-rich phase for development.

Solvent III: Cyclohexane and a 5:1 mixture of dimethylformamide and pyridine were equilibrated in a separatory funnel, and the layers were distributed in the chromatographic chamber as described under *Solvent II*. The chromatograms were suspended in the chamber 6-8 hours for equilibration and were then lowered into the cyclohexane-rich layer for development.

Solvent IV: The solvent was prepared by equilibrating cyclohexane and a 3:2 mixture for dimethylformamide and pyridine. The remainder of the procedure was identical to that described under *Solvent III*.

Chromatographic procedure, acidic carbonyl derivatives. The procedure was essentially the same as that described for the neutral derivatives, except that the deriva-

TABLE 2

Neutral carbonyl compounds
Paper chromatography of the 2,4-dinitrophenylhydrazine derivatives

Compound	Rf Values[1]				Color in NH_3
	Solvent I	Solvent II	Solvent III	Solvent IV	
Formaldehyde	0.23	0.09	0.29	0.46	Yellow-brown
Acetaldehyde	.36	.30	.42	.57	Yellow
Propionaldehyde	.50	.44	.55	.74	Yellow
Propanone	.50	.38	.50	.69	Yellow
2-Butanone	.62	.53	.66	.78	Yellow
2-Pentanone	.70	.60	.78	.90	Yellow
2-Octanone	.86	.78	.95	.97	Yellow
Acetoin	.24	.20	.29	.49	Red-violet
Diacetyl	.24	.20	.29	.48	Red-violet
Acetylmethyl acetate	.29	.18	.24	.42	Yellow
Diacetone alcohol	.45	.27	.71	.80	Yellow
Vanillin	Streak	.02	.03	.10	Orange
Salicycladehyde	Streak	.07	.10	.21	Red-orange
Furfural	Streak	.14	.17	.30	Orange-brown
Cinnamaldehyde	Streak	.24	.28	.43	Yellow-orange
Benzaldehyde	Streak	.27	.32	.48	Yellow-orange
Anisaldehyde	Streak	.15	.19	.35	Yellow-orange
Methyl p-tolyl Ketone	—	.33	.47	.60	Yellow
2, 4-Dinitrophenylhydrazine	—	—	.59	.70	

[1] Solvent I: Heptane equilibrated against methanol. Solvent II: Cyclehexane equilibrated against dimethylformamide. Solvent III: Cyclohexane equilibrated against dimethylformamide/pyridine (5/1). Solvent IV: Cyclohexane equilibrated against dimethylformamide/pyridine (3/2).

TABLE 3

Neutral carbonyl compounds in roasted coffee
Paper chromatography of the 2,4-dinitrophenylhydrazine derivatives

Band	Rf Value[1]		Color in NH_3	Relative Intensity	Presence in green coffee	Tentative Identity
	Solvent II	Solvent IV				
1)	0.72	0.85	Yellow	+		Aliphatic aldehydes or Ketones (>C_5)
2)	.64	—	Yellow	+		
3)	.37	.49	Red-violet	+	−	(Dicarbonyl ?)
4)	.35	.47	Yellow-brown	+++	+	?
5)	.22	.37	Red-violet	+	−	Acetoin and/or diacetyl
6)	.21	.35	Yellow-orange	+++	−	?
7)	.13	.30	Orange-brown	++++	−	Furfural
8)	.10	.26	Red-violet	+++	−	(Dicarbonyl ?)
9)	.05	.22	Yellow-orange	+	+	?
10)	.03	.10	Brown	++++	−	Product of Glucose Browning
11)	—	.07	Violet-brown	+	?	Vanillin
12)	.01	.05	Brown	+++++	−	Product of Glucose Browning
13)	—	.03	Violet-brown	++	?	?
14)	.00	.00	Brown	+++++	+	Hexoses, etc.

[1] Solvent II: Cyclohexane equilibrated against dimethylformamide. Solvent IV: Cyclohexane equilibrated against dimethylformamide/pyridine (3/2).

tives were dissolved in a minimum amount of chloroform for application to the paper. The following solvent system was devised: A mixture of *t*-butanol/butanone/heptane (1/1/1, v/v/v) was equilibrated against 1.5 N ammonium hydroxide. The chromatograms were suspended for three hours in an atmosphere containing the aqueous phase and were then developed with the organic phase.

RESULTS AND DISCUSSION

The results of this study are shown in Tables 2-5 and indicate a complex array of carbonyl compounds. The dimethylformamide containing solvents provided good separation of the neutral carbonyl derivatives and tolerated relatively heavy applications of the samples. A total of 14 bands were distinguished when these solvents were applied to roasted coffee.

TABLE 4

Acidic carbonyl compounds
Paper chromatography of the 2,4-dinitrophenylhydrazine derivatives
Solvent: t-butanol 1/2-butanone/heptane (1/1/1, v/v/v), equilibrated against 1.5 N ammonium hydroxide.

Acid	Rf Value	
Oxalsuccinic	0.99	
Acetoacetic	.98	
Hydroxypyruvic	.97	
Phenylpyruvic	.80	.52
α-Keto-*n*-Caproic	.80	.52
α-Keto-*n*-valeric	.73	.39
Oxalacetic	.17	.03
Mesoxalic	.33	.02
β-Mercaptopyruvic	.30	

TABLE 5

Acidic carbonyl compounds in green and roasted coffees
Paper chromatography of the 2,4-dinitrophenylhydrazine derivatives
Solvent: t-butanol 1/2-butanone/heptane (1/1/1, v/v/v), equilibrated against 1.5 N ammonium hydroxide.

	Rf Value	Relative Intensity
Roasted coffee:		
(1)	0.97	+++
(2)	.83	+
(3)	.60	++
(4)	.42	+++
(5)	.28	++++
(6)	.20	++
(7)	.11	+++++
(8)	.02	+++
(9)	.00	+++
Green coffee:		
(1)	0.99	+++++
(2)	.84	+
(3)	.35	+++
(4)	.23	+
(5)	.09	++++
(6)	.05	++
(7)	.02	+
(8)	.00	++++

Solvent I produced 8 bands, but demonstrated a low capacity and poor separation, and the R_f values are not included. It is to be noted that the addition of pyridine to the cyclohexane-dimethylformamide system results in increased R_f values and better separation of the slower-moving derivatives.

Some tentative conclusions regarding the neutral carbonyl compounds are listed in Table 3. These conclusions are not based on R_f values as such, but on the results of many chromatograms spotted with various combinations of known compounds together with the coffee mixtures. Six bands predominated, and one of these was indicated to be furfural. Two of the heavy bands did not correspond to those of any known derivatives, but did correspond to the 2 bands which were obtained from a sample of glucose which had been slightly browned over a flame. One weak band corresponded to that of diacetyl and acetoin and another weak band coincided with that of vanillin. Green coffee produced 4 bands, and those which were common to both green and roasted coffee are indicated. The only evidence of simple aliphatic aldehydes and ketones was the presence of 2 weak bands having R_f values in the C_5-C_8 range.

No definite conclusions can be drawn from the data regarding the acidic carbonyl compounds in Tables 4 and 5. The new solvent system which was developed resulted in excellent separation of the acidic carbonyl derivatives from roasted coffee, producing 9 distinct bands. However, thorough study will require a more elaborate array of known compounds. In any case, the limitations of the method must be realized, since it has been shown that the reaction of keto-acids with 2, 4-dimethylphenylhydrazine results in isomers giving multiple spots (*4*).

Positive identification of the various carbonyl compounds in roasted coffee remains to be accomplished. However, paper chromatography appears to offer a satisfactory means for separation and tentative identification, and produces characteristic and reproducible patterns. Elution followed by spectrophotometric measurements should lead to further characterization and quantitative assay.

Precipitated Material in Acidified Coffee Brew

Acidification of brewed coffee results in a flocculent brown precipitate. During preliminary procedures it was noted that cold-water extracts fail to yield this material. A few exploratory experiments were carried out to determine the nature of this precipitate, with a view toward eliminating it from extracts to be chromatographed.

The substance was found to be soluble in alkali, including ammonia, and in certain organic solvents such as glacial acetic acid, but insoluble in water or aqueous acids. It was also noted that the substance was reprecipitated from alkaline solution upon acidification. A small amount of the material was isolated and partially purified, and the acid hydrolysate of the material was subjected to paper chromatography for the determination of possible amino acids.

EXPERIMENTAL

Preparation of precipitated material. A suspension of 100-150 g. ground roasted coffee in one liter of distilled water was heated over a steam bath for 30 minutes, and the mixture was filtered. The extract was poured into dilute hydrochloric acid; the precipitate was removed by centrifugation; and the supernatant was discarded. The material was redissolved, reprecipitated, then washed until free of hydrochloric acid, and finally dried over calcium chloride.

Hydrolysis. A small amount, 20-50 mg., of the material was refluxed with 5 ml. 6 N hydrochloric acid for 48 hours. The mixture was filtered, and the filtrate was neutralized and desalted on an ion-exchange column using Dowex-50. The solution was finally concentrated to a syrup for application to chromatograms.

Paper chromatography. The procedure was similar to that of Block (*2*). Desalted hydrolysate was spotted on 8 X 8 inch pieces Whatman No. 1 paper with a cut-off No. 22 hypodermic needle, to give spots approximately 2 mm. in diameter. Known amino acids were also spotted on the sheets. The chromatograms were suspended 12 hours over 3% ammonium hydroxide (with potassium cyanide as described by Block [*2*]), and developed with ascending 80% phenol. The chromatograms were air-dried for 12 hours and sprayed with 1% ninhydrin in 1:1 n-butanol: acetone. After evaporation of the acetone, the chromatograms were heated to 90° C. for color development.

RESULTS AND DISCUSSION

At least 10 amino acids appeared in the hydrolysate (Table 6). Hydroxyproline was qualitatively identified in addition to those reported by Underwood and Deatherage (*11*). A large portion of the material failed to go into solution on refluxing, and no study was made of this residue.

No flavor was detected in the purified precipitate. It could be a factor in the color of the brewed coffee, however, since it exhibits a deep brown color when in solution, and apparently the pH of freshly brewed coffee favors solution of the substance. It is obviously involved in the relationship between pH and turbidity. The substance may also exert an important buffering effect.

TABLE 6

Amino acids in the brown precipitate from acidified coffee brew
Paper chromatography of the acid hydrolysate
Solvent: Phenol in 3% NH_3 atmosphere. Indicator: Ninhydrin

Amino Acid	Rf	Color
(1)	0.97	Brown-purple
(2)	.94	Purple
(3)	.88	Purple
(4)	.77	Pink-purple
(5)	.67	Yellow-brown
(6)	.56	Purple
(7)	.45	Pink-purple
(8)	.22	Purple
(9)	.09	Blue
Leucine	.86	Purple
Isoleucine	.87	Purple
Valine	.76	Purple
Hydroxyproline	.66	Yellow-brown
Alanine	.55	Purple
Threonine	.47	Pink-purple
Glycine	.41	Pink-purple
Glutamic acid	.19	Purple
Aspartic acid	.12	Blue

SUMMARY

Chromatographic methods were applied to a study of the non-volatile acids, volatile acids, and acidic and neutral carbonyl compounds in green and roasted coffee, and to a study of acid-precipitated matter in coffee brew. In general, the results were tentative, but indicate that an intensive application of the procedures could provide much information regarding flavor constituents of roasted coffee.

Ten non-volatile acidic constituents were revealed by paper chromatography. Seven of the compounds fluoresced under ultraviolet light, and at least six of the compounds were indicated to be phenolic.

A buffered silica-gel column showed the presence of five volatile acids in significant amounts. Four were identified tentatively as valeric, butyric, propionic, and acetic acids. A fifth acid did not correspond to any simple aliphatic acid. Acetic acid appeared to predominate.

Three new solvent mixtures were developed for paper chromatography of the neutral carbonyl compounds, and at least fourteen were distinguished. One new solvent system was developed for chromatography of the acidic carbonyl compounds, and revealed nine bands.

A quantity of the acid-precipitated material in coffee brew was prepared and partially purified. Paper chromatography of the acid hydrolysate revealed the presence of at least nine amino acids.

Acknowledgments

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FLAVORS IN FOODS—A CUMULATIVE REVIEW

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I. GENERAL PLAN OF THE REVIEW SERIES

In a previous number of *Food Research (1)* a cumulative review of the flavors in foods was suggested. In order to obtain proper coverage, the authors of articles, reviews, and monographs were invited to share in the cumulative review by providing summaries of articles which are of interest to the field of flavor research. Critiques were also suggested as a measure of progress which a certain endeavor may have made over a selected period of time. It now appears that the latter suggestion is the line of development to follow. At the suggestion of the editor, the author has prepared a prospectus of discussions for this latter, narrative type of review. Steps have been taken to implement the suggestion and the purpose of the present paper is to outline the concept and general plan.

The decision to turn from abstracts to the narrative type review was reached after realization that abstracts are useful only if full coverage of the world's literature can be made. It was also recognized that full coverage would be an idealistic dream if attempted by voluntary contributions alone. It seemed much more possible to achieve the desired results by means of narrative reviews of particular segments of flavor research. Prepared by a recognized authority, such a review will provide provocative thought and stimuli to a definable area of this rather broad field of investigation.

CATEGORIES OF FLAVOR RESEARCH

The categorization of any field is necessarily arbitrary. The author, in offering his version of the most useful classification of flavor research, is not unaware of the many possible divisions of this broad field. He has settled upon commodity groups and techniques in preference to a more basic approach on grounds that better unity and continuity might be obtained in a given article and that it would increase the value of the series for reference work.

It is suggested that one article be covered in each number of *Food Research* and that the reviews might be renewed biennially. The following are intended only as titles, no chronological order being implied:

1. Chemical and Physical Methods.
2. Meat, Poultry and Fish Flavors.
3. Flavors in Dairy Products.
4. Flavors in Vegetables and Vegetable Juices.
5. Flavors in Fruits and Fruit Juices.
6. Flavors of Cereal Products.
7. Flavors in Coffee, Tea and Chocolate.
8. Flavors of Alcoholic Beverages.
9. Spice and Condiment Flavors.
10. Psychology of Flavor and Methods of Testing.
11. Physiology of Flavor.

A twelfth category has been purposely omitted to allow either an inclusion of a neglected category, or a general review and discussion of the biennium in flavor research.

SCOPE OF THE REVIEWS

Inasmuch as the reviews are to be renewed every other year, it is expected that the majority of references will be to current investigations. By limiting the reviews to a commodity class, the coverage of the literature given by the reviewer should be most comprehensive.

Invitations for authorship will be sent to authorities in each field of research in the name of the editor of *Food Research* and subject to approval of the editorial board. Reviews submitted by invitation will be subject to the same editorial considerations as research papers including consideration of criticism of impartial manuscript reviewers. Voluntary contributions will be given full consideration but to prevent confusion it is requested that those who wish to contribute correspond with the editor before preparing an article.

It is hoped that this narrative type review article can commence with the May-June number of *Food Research*. Consideration of the proposed authors will determine the chronological order of the above categories. Suggestions as to the improvement of the plan will always be welcome.

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II. SELECTED SUMMARIES OF RECENT LITERATURE

In order to preserve continuity with the initial article, selected abstracts are included below.

Chemical Aspects of Flavor

Strawberry flavors. M. B. Jacobs. *Am. Perfumer and Aromatics*, **68**, 53 (1956). A review of recent contributions to the chemistry of natural strawberry flavor components. Synthetic compounds and typical formulations for imitation strawberry flavor are given. In contrast to earlier formulations comprising 8 or 10 ingredients, a more recent one uses 32.

Vanilla extracts and imitations. Determination of vanillin, ethyl vanillin, and coumarin by partition chromatography and spectrophotometry. L. G. Ensminger, *J. Assoc. Offic. Agr. Chemists*, **39**, 715 (1956). Refinements in previously published methods and their application are given. Methods for the analysis of bakery products are emphasized.

Stabilizing the flavor of essential oils. J. W. Evans and A. W. Kleinschmidt (to American Maize-Products Co.) U. S. 2,754,215. July 10, 1956. A flavoring composition is prepared by making an emulsion consisting of water and droplets of an oil which carries the flavor and which droplets are coated with a protein material. Such coating serves to stabilize the flavor and prevent oxidation deterioration. Examples and directions for preparing such emulsions are given.

Studies on protein hydrolysis. IV. Further observations on the taste of enzymatic protein hydrolyates. J. W. Carr, T. C. Loughheed, and B. E. Baker. *J. Sci. Food Agr.*, **7**, 629 (1956). Enzymic hydrolyzates were prepared from alpha-, beta-, and gamma-fractions of the casein complex by use of trypsin, and from commercial muriatic by the use of crystalline trypsin and crystalline chymotrypsin. These hydrolyzates had a bitter taste. A series of fractionations of a tryptic casein hydrolyzate yielded a product that had an extremely bitter taste. Chromatographic and electrophoretic studies have suggested that this material was a single polypeptide. Use of 2:4-dinitrofluorobenzene failed to reveal the presence of N-terminal amino-acids; the material yielded leucine, valine, and glumatic acid on hydrolysis with carboxy-peptidase.

Occurrence of a bitter principal in carrots. A. Dodson, H. N. Fukui, C. C. Ball, R. L. Carolus, and H. M. Sell. *Science*, **124**, 974 (1956). Bitter carrots were dried and extracted with acetone. Bitter crystals, which separated from solution on removal of the acetone, were recrystallized from aqueous methanol. Analytical data suggested that the compound has the empirical formula of $C_{15}H_{13}O_5$. No compound in the literature could be found with properties corresponding to the bitter compound.

Lactones for flavoring margarine. J. Boldingh, P. H. Begemann, G. Lardelli, R. J. Taylor and W. T. Weller (to Unilever Ltd.). Brit. 748,661, May 9, 1956. Delta-dodecalactone is useful for imparting a butter-like flavor to foods. A method for synthesizing the compound from the starting materials, cyclopentanone and hexanal, is given.

Ibid. R. J. Taylor and W. T. Weller (to Unilever Ltd.). Brit. 748,801, May 9, 1956. Epsilon-lactones of saturated normal aliphatic acids containing 9 to 14 carbon atoms are useful for imparting butter-like flavor to foods. A method of synthesis starting with cyclohexanone and various aliphatic aldehydes is given.

Acceptability of irradiated foods. II. V. E. McGary and M. E. Shipman. *J. Am. Dietet. Assoc.*, **32**, 1059 (1956). Irradiated foods were fed to human beings in 3-balance studies. Approximately 35, 65 and 80 per cent of the diet was supplied by irradiated foods. Acceptability data was obtained by questionnaire and interviews. Many irradiated foods were as acceptable as non-irradiated control foods. Irradiated fish, meats and fruits were well received by most subjects.

A chemical and flavor study of off-flavor due to artificial pepper. E. L. Wick and S. E. Cairncross. *Food Technol.* **10**, 423 (1956). A black pepper oleoresin unsuitable for food use was found to be low in piperine and to contain an unnatural component, 1-cinnamoyl piperidine. The volatile portion contained a sesquiterpene hydrocarbon fraction, which was responsible for the off-flavor.

Physiology of Flavor

Olfactory and trigeminal nerve responses to odors. L. M. Beidler and D. Tucker. *Fed. Proc.*, **15**, 14 (1956). Olfactory nerve activity of rabbit showed no adaptation to low concentrations of odors and complete adaptation to high concentrations. The response was dependent upon flow rate of odor through the nose.

Observations on the structure and organization of olfactory receptors in the rabbit. W. E. L. Clark. *Yale J. Biol. Med.*, **29**, 83 (1956). Microscopical observations of rabbit olfactory receptors show variations in length of rod processes, size of terminal swellings, number of olfactory hairs and affinity for silver stains. Most receptors degenerate after bulbar lesion.

Olfactory nerve fibers. H. S. Gassner. *J. Gen. Physiol.*, **39**, 473 (1956). An extensive electron microscope study of the olfactory receptors of pigs indicates a 0.2 u diameter for the nerve fibers. The fibers are organized into larger bundles in an unusual manner as they go from the receptors to the bulb. The conduction velocity of the olfactory nerve of the pig is 0.2 m/sec. Some aspects of the cytology of the bipolar and sustentacular cells are described.

Analysis of the electrical activity of the olfactory epithelium. D. Ottoson. *Acta Physiol. Scand.*, **35**, Supp. 122 (1956). The response of the olfactory epithelium of the frog to odors is obtained by measuring the change in electrical potential across the olfactory epithelium initiated by the olfactory receptors. The change in potential with stimulus intensity, odor volume, time course of stimulus and molecular configuration of odor is discussed.

Smell—The physical sense. A. W. Middleton. *Perfumery Essent. Oil Record*, **47**, 201 (1956). The sense of smell is shown to be analogous to that of hearing and to be a physical sense. The fundamental physiology or physical basis of the different olfactory phenomena is explained and the explanations are suitable criteria for the assessment of experimental work.

The part played by taste and smell in infant feeding. M. R. Du Pan. *Pediatrie* **10**, 169 (1955); *Nutrition Abstr. & Rev.* **26**, No. 930 (1956). Reaction to solutions of sugar and of salt was investigated in 20 infants aged from 3 days to 8 weeks. Even at one week infants showed preference for sugar, and strong reactions to taste were apparent in all infants more than a few days old. Olfactory and taste reactions were studied with vegetables in infants aged from 3 to 8 months. Carrots were preferred to tomatoes, peas, haricot beans, or mixed vegetables. In view of the positive findings on reactions to taste and smell in very young children, it is considered that more attention should be given to palatability in planning diets for infants.

NOTES AND LETTERS

RELATION OF METHIONAL TO THE ODOR OF IRRADIATED MEAT[a, b]

Several compounds related to methionine have been identified as flavor and odor principles in foodstuffs. Haagen-Smit *et al.* (*4*) found methyl 3-methylthiopropionate in pineapples; Patton (*8*) reported that methional (3-methylthiopropionaldehyde) was responsible for the sunlight flavor in milk. Recently, Patton (*9*) stated that a variety of cooked foods containing methionine develop an odor reminiscent of methional and the compound may be a rather general flavor and odor principal.

Research on the flavor and odor of gamma-irradiated meat has led to the detection of mercaptans (*1*), α, β-unsaturated aldehydes, and lipide peroxides in the meat. In this laboratory we have generally found that about 30% of all carbonyl compounds produced by the irradiation of a typical meat fat (lard) were α, β-unsaturated. In the presence of a peroxidic catalyst, acetyl peroxide (*7*), benzoyl peroxide, or ascaridole (2), methyl mercaptan has been shown to condense with acrolein to give a high yield of methional. Work was therefore undertaken to determine whether this condensation product, or related materials, were to any extent responsible for the odor noted in irradiated meat. Six batches of methional and several batches of the next higher homologue, 3-methylthiobutyraldehyde, were prepared. Methyl 3-methylthiopropionate was prepared by the method of Hurd and Gershbein (*5*) and also by direct treatment of methional with 30% hydrogen peroxide followed by esterification.

The crude reaction mixture containing methional had an odor typical of ground beef which had received 2-4 megarep of gamma radiation, while the mixture containing 3-methylthiobutyraldehyde had an odor typical of meat at higher radiation dosages (8-10 megarep). Physical constants for these compounds were in agreement with published values, except for the 2,4-dinitrophenylhydrazone of 3-methylthiobutyraldehyde. Repeated recrystallization of the chromatographically pure derivative failed to raise the melting point (75-6° C.) to the reported value of 85-6° C. (*2*). Microcombustion, however, substantiated the formula, $C_{11}H_{14}N_4O_4S$. Calculated: C - 44.50%; H - 4.60%: found: C - 44.30%; H - 4.73%.

Purification of the aldehydes by vacuum distillation through an 18-cm. Vigreaux column invariably resulted in *odorless* materials. After distillation both of the aldehydes rapidly develop offensive odors, presumably as the result of air oxidation. Within 3-5 minutes a distinct odor was noted, which increased in intensity upon prolonged exposure to air. The ester (methyl 3-methylthiopropionate) has a mild pineapple odor when fresh, but developed a rather disagreeable odor after exposure to air.

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[a] Report of research sponsored by the Quartermaster Food and Container Institute for the Armed Forces, assigned Number 705 in the series of papers approved for publication. Views or conclusions contained in this report are those of the authors and are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

[b] Journal Paper No. 138, American Meat Institute Foundation.

Oxidation at the thio ether linkage seemed necessary for the appearance of the odor attributed by Patton to methional. Although methional may be the cause of the sunlight flavor in milk, it definitely is not, *per se*, the source of odor in irradiated meat. It may, however, give rise to odoriferous compounds on oxidation. The sulfone of the ester has been described previously (*4*) and the sulfone of the diethylacetal of methional was prepared in this laboratory. These materials are crystalline solids melting at 94° and 142° C., respectively. Preliminary research data suggest that the sulfoxide is the odoriferous product present in highly purified methional preparations after exposure to air. This would be comparable to the series methionine, methionine sulfoxide, methionine sulfone, wherein the sulfoxide is the member with the noteworthy aroma (*6*).

The occurrence of methional and/or related compounds in gamma-irradiated meat would present a peculiar situation. Withdrawal of such odorous materials as methyl mercaptan and acrolein into an odorless condensation product would be desirable if the product were not itself so unstable. Besides the oxidative instability of methional, the condensation reaction is reversible under alkaline conditions, regenerating the mercaptan and the aldehyde. If methional were transmethylated in a biological media to dimethylpropiothetin, the enzymatic degradation of this compound to dimethyl sulfide and acrolein is a known reaction (*3*).

Work is in progress to elucidate more clearly the role of methional and related compounds as flavor and odor principals in foodstuffs.

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